A Study of the Interactors of Eps15 Homology Domain and the Role of the EH Network in the Model System of *Caenohabditis elegans*

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A Study of the Interactors of Eps15 Homology Domain and the Role of the EH network in the Model System of *Caenorhabditis elegans*

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LIST OF ABBREVIATIONS

EH: Eps15 Homology

EGFR: Epidermal Growth Factor Receptor
Eps15: EGFR pathway substrate protein 15
EHD: Eps15 Homology Domain-containing proteins
Reps: RalBP1-Associated Eps15 homology proteins

AP: Adaptor protein complex
ESCRT: Endosomal Sorting Complex Required for Transport)
AC: Adenylate Cyclase
GPCR: G-protein coupled Receptor
APP: Amyloid Precursor Protein

CCV: clathrin-coated vesicles
MVB: Multivesicular bodies

NPF: asparagine-proline-phenylalanine
FW: phenylalanine-tryptophan
WW: tryptophan-tryptophan
HTF: histidine-threonine-phenylalanine
HSF: histidine-serine-phenylalanine
DPF aspartic acid-proline-phenylalanine

Y2H: Yeast Two Hybrid

C. elegans: *Caenorhabditis elegans*
S. cerevisiae: *Saccharomyces cerevisiae*
D. melanogaster: Drosophila melanogaster

AD: Alzheimer's Disease

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ABSTRACT

Eps15 homology (EH) domain-containing proteins have been implicated in diverse intracellular signalling pathways, such as endocytosis, actin cytoskeleton organization, nucleo-cytosolic shuttling and mitogenic signalling. However, the extent of the protein-protein interactions mediated by the EH domain at the level of a whole organism has not yet been addressed. This project aims to gain an overview of the EH network in a model system, *C. elegans*, by identifying interactors of all EH-domain containing proteins present in *C. elegans*. Five genes encode EH proteins in the *C. elegans* genome and the isolated EH domains were used to screen a *C. elegans* cDNA library using the Yeast Two Hybrid system. The validation of the putative interactions was carried out by *in vitro* pull-down assays. The biological relevance of the interactions was tested genetically using *C. elegans* as a model system. The genetic interactions were monitored using available mutants for four of the five EH encoding genes, in which the genes of the putative interactors were knocked-down by RNA *interference*. A total of 26 proteins were found to interact physically and genetically with at least one EH protein. Most of the interactions identified are novel and highlight new pathways in which the EH network is involved. The results of this study indicate that the EH complexes can be found in various intracellular compartments, holding together a network of adaptor proteins involved in all the major processes of the protein sorting events during intracellular signalling.

I. INTRODUCTION

In the first part of the introduction, the identification of the EH domain, its structure and its interacting motif will be discussed. Four major EH-domain containing protein families have been identified to date, namely; the Eps15 family, the Intersectin family, the EHD family and the Reps family. Each EH protein family will be introduced by exploring the current knowledge of the family members found across the species from yeast to mammals.

The subsequent part of the introduction will focus on the experimental approaches that were taken in this study. This project aimed to perform a genome-wide screening to identify the interactors of all *Caenorhabditis elegans* (*C. elegans*) EH proteins, using the Yeast Two Hybrid (Y2H) technique. The interactions discovered through the Y2H screenings were validated by *in vitro* binding, followed by genetic studies using *C. elegans*. Therefore, the first section in the experimental approaches will elaborate on several reasons why *C. elegans* is an ideal model system for this study. Secondly, the advantages and disadvantages of the Y2H system will be examined, especially in the context of genome-wide screenings.

The final part of the introduction will aim to summarize the existing picture of the EH network, and how this project can contribute to analyse the EH network in a whole organism.

1. The Eps15 Homology (EH) Domain

1.1. Identification of the evolutionarily-conserved EH domain

The Eps15 Homology (EH) domain was first identified as a repeated domain present in the N-terminal portion of Eps15 (Epidermal growth factor receptor pathway substrate #15), a substrate of the tyrosine kinase domain of the epidermal growth factor receptor (EGFR) [1].

The EH domain is a protein-protein interaction domain of about 70 amino acids (Figure A), present in 3 copies at the N-terminal portion of Eps15 and EPS15 Related protein (EPS15R). The EH domain has been conserved through evolution, as sequences homologous to this domain are found in the genome of *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Mus musuculus*, and *Homo sapiens* [2].

Apart from a few EH proteins that do not appear to have obvious homologues in other species, such as yeast EH proteins YKR019cp/Irs4p and YJL083wp, and the mammalian γ–Synergin (even though putative homologues in *C. elegans* and other species have been identified recently, as described in the following section 2.5), most of them were found to belong to four major families of EH proteins. The main EH proteins families are: the Eps15 family, the Intersectin family, the EHD (Eps15-Homology-Domain containing proteins) family and the Reps (RalBP1-Associated Eps15 homology proteins) family. The structures of these major families of EH proteins are shown in Figure B. (For more detailed descriptions of other EH proteins found in yeast, refer to the review by Santolini *et.al.* [3])

Figure A The conserved sequences of Eps15 Homology Domains in Eps15 and Eps15R
(The figure was modified from article by Wong *et.al.* [4])

Eps15-/Eps15R-EH1: the amino acid sequences of the first EH domains of Eps15 and Eps15R

Eps15-/Eps15R-EH2: the amino acid sequences of the second EH domains of Eps15 and Eps15R

Eps15-/Eps15R-EH3: the amino acid sequences third EH domains of Eps15 and Eps15R.

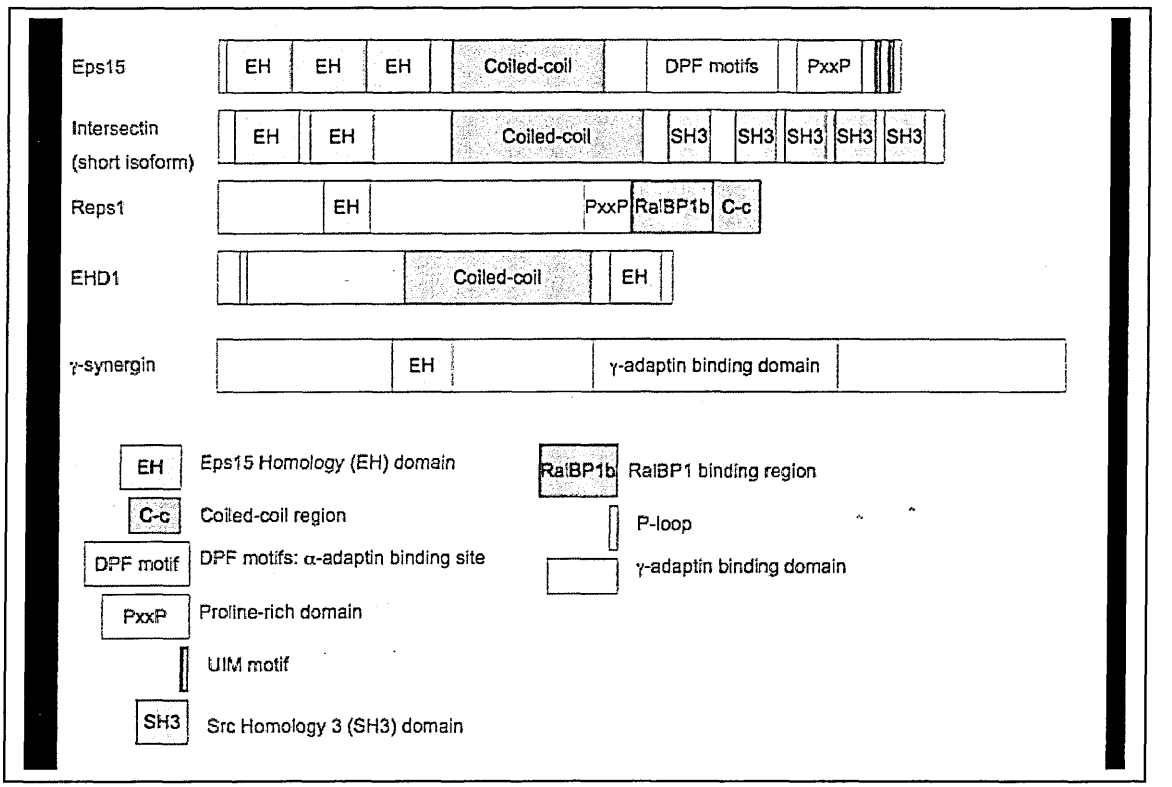
The Consensus sequence of EH domain; a: acidic residue, b: basic residue, ϕ: hydrophobic residue and -: any residue.

```
            1                                                                70
Eps15-EH1   GNPVYEKYYRQVDTGNTGRVLASDAAAFLKKSGLPDLILGKIWDLADTDGKGILNKQEFFVALRLVACAQ
Eps15R-EH1  GNSLYESYYKQVDPAYTGRVGASEAALFLKKSGLSDIILGKIWDLADPEGKGFLDKQGFYVALRLVACAQ

            1                                                                70
Eps15-EH2   DKAKYDAIFDSLSPVNGFLSGDKVKPVLLNSKLPVDILGRVWELSDIDHDGMLDRDEFAVAMFLVYCALE
Eps15R-EH2  EKAKFDGIFESLLPINGLLSGDKVKPVLMNSKLPLDVLGRVWDLSDIDKDGHLDRDEFAVAMHLVYRALE

            1                                                                70
Eps15-EH3   EKAKYDEIFLKTDKDMDGFVSGLEVREIFLKTGLPSTLLAHIWSLCDTKDCGKLSKDQFALAFHLISQKL
Eps15R-EH3  DKMRFDEIFLKTDLDLDGYVSGQEVKEIFMHSGLTQNLLAHIWALADTRQTGKLSKDQFALAMYFIQQKV

Consensus   aK--ϕa-IF---a----G-V-G--Vb-ϕϕϕ-SGLP--ϕL-bIW-L-D-a--G-L-b-EFAϕAMbL
```

1.2 The structure of EH domain

The NMR structure of the second EH domain of Eps15 revealed that it consists of four alpha helices; αA, αB, αC, and αD, where αA and αB are connected by a β-sheet, and αC and αD are connected by another β-sheet that is positioned in an anti-parallel manner with respect to the first β–sheet [5] (Figure C). The EH domain was found to contain two EF-hand motifs, a motif which acts as a calcium-binding region. Even though calcium-binding often acts as a regulatory mechanism for various proteins, the addition of calcium did not alter the ability of the EH domains to interact with proteins significantly [4]. In addition, it was shown that the EF-hands of Eps15 display different affinities and positions for calcium-binding [5]. Therefore calcium-binding to the EH domain is suggested to have a structural role. The structures of various EH domains have been solved to date, such as the EH domain of POB1 [6] and Reps1 [7, 8], the first EH domain of Eps15 [9] and the third EH domain of Eps15 [10]. These studies demonstrated that all EH domains share the same conformation.

Figure B The Structures of EH domain-containing proteins found conserved through evolution. (Adapted from the review by Santolini et al. [3])



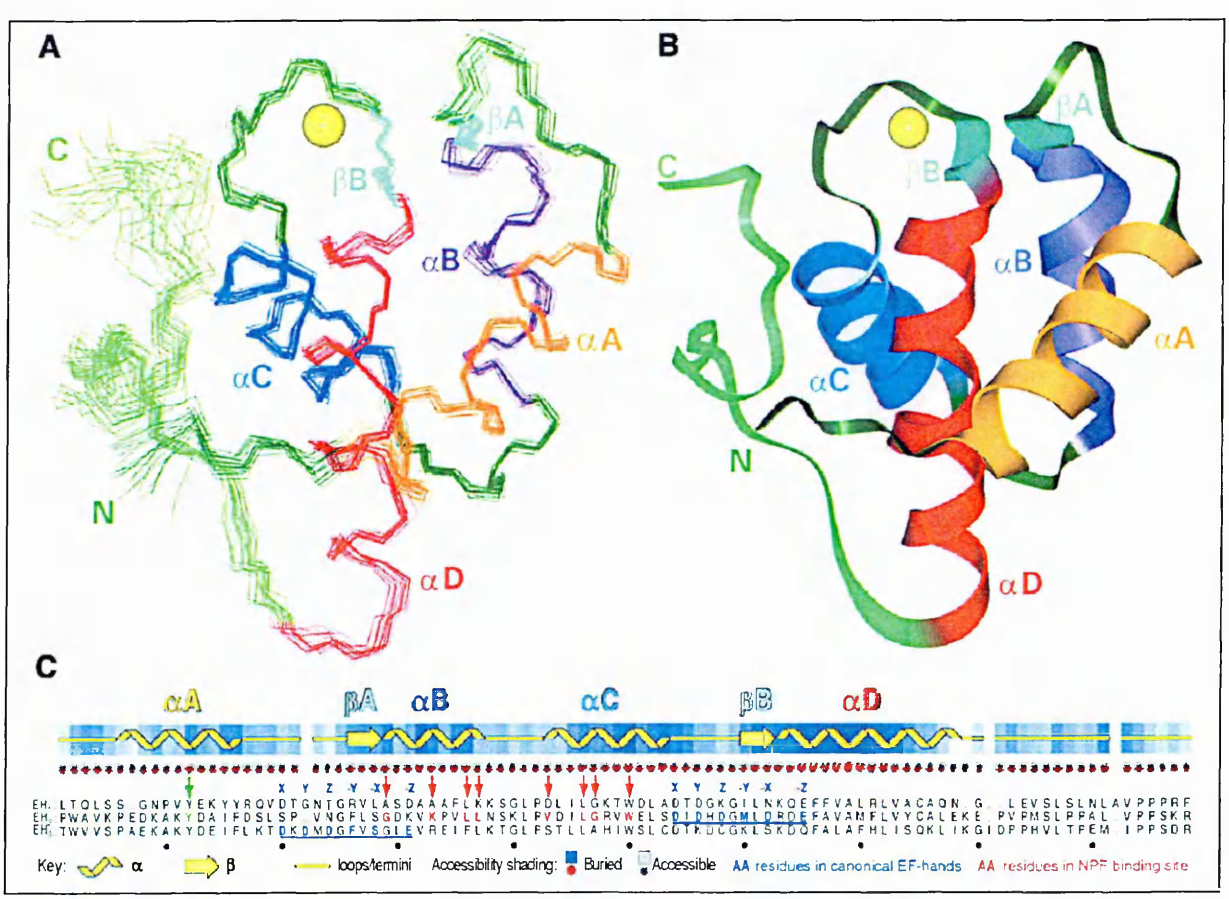
1.3 Motifs that bind to the EH domain

A tripeptide motif of NPF (asparagine-proline-phenylalanine) was highlighted as the common interaction motif as a result of a screening of a random phage-displayed peptide library using the GST-fusion proteins of the EH domains of Eps15 and Eps15R [11]. The NPF motif was found in 46 out of 48 peptides that interacted with the EH domains, whereas the remaining two peptide sequences selected by the EH domain of Eps15R contained either NHF (asparagines-histidine-phenylalanine) or HPF (Histidine-proline-phenylalanine). In this study, it was also shown that a single EH domain is sufficient to mediate protein-protein interactions [11].

Further investigation on the EH-interacting regions using eleven different EH domains confirmed the NPF tripeptide (Class I) as the most common interactive motif in addition

to two more classes of peptides [11]. Class II peptides identified from the phage-display experiments with various EH domains contain dipeptide motif FW (phenylalanine-tryptophan) or WW (tryptophan-tryptophan), and a tripeptide motif of SWG (serine-tryptophan-glycine). The third class of EH interacting motifs were tripeptides: HSF (histidine-serine-phenylalanine) or HTF, (histidine-threonine-phenylalanine). These motifs are recognized exclusively by the first EH domain of a yeast EH protein, End3p [12].

Figure C Structure and Asn-Pro-Phe (NPF) binding pocket of the Eps15 homology domain (Taken from the article by de Beer et al. [5])
A: The best-fit superposition of the backbone atoms (N, Cα, and C') in the secondary structure elements of the 20 structures with the lowest nuclear Overhauser effect energies
B: The ribbon diagram of the structure closest to the average of the 20 structures depicted in A.
C: The amino acid sequence alignment of the three EH domains of human Eps15.
The yellow spheres in the three dimensional structures (A & B)indicate the position of the calcium ions (Ca2+) in the EF-hand-like calcium-binding pockets. The pink arrows pointing to the residues in the amino acid sequence (C) indicate the NPF-motif binding residues.



Structural studies confirmed that NPF peptides bind the hydrophobic surface in the EH domain [5], and that the type I β-turn conformation of NPF peptides is buried in between two α-helices of the EH domain [7]. The specificity of the interaction mediated by the hydrophobic pocket of the EH domain was further demonstrated when the disruption of the hydrophobic surface by a point mutation was shown to alter the interaction affinity [10].

To date, several interactors of EH domains that contain the NPF motifs have been identified, such as Hrb, Hrb-related protein Hrb-l, Numb and Numb-related protein, Numb-l [11], Epsin [13-16], TTP [17]. Most of the interactors that have been characterized to date are summarized in Table A, which was taken from the review by Polo *et al.* [18].

2. The EH network

The function of the EH network can be better appreciated by considering the properties of EH-containing proteins and their interactors. The EH network was first implicated in the process of endocytosis at the plasma membrane. Some EH-containing proteins were also involved in intracellular trafficking, whilst some others play a role in the regulation of actin cytoskeleton organization. Finally, on the basis of known interactions, the EH proteins may also participate in different processes, such as mitogenic signalling, cell proliferation and nuclear shuttling.

Numerous studies of each family of EH-domain-containing proteins in mammalian cells, as well as in *Xenopus laevis (X. laevis)*, *Drosophila melanogaster (D. melanogaster)* and in *C. elegans*, have provided insights into a network of protein interactions that are implicated in diverse intracellular signalling pathways. The following sections on the EH-containing protein families will describe their functions and highlight the networks in which these proteins are engaged.

2.1 Eps15 family

2.1.1 Introduction of the Eps15 family

The Eps15 protein was identified in a screening for substrates of the EGFR tyrosine kinase domain as well as of another tyrosine kinase receptor, platelet-derived growth factor receptor (PDGFR). [1]. A screening of the mouse keratinocyte cDNA library with the EH-domain containing region of Eps15 identified an Eps15-related gene, Eps15R, which has been mentioned in Section 1.1 [4]. As evident from the sequence alignment shown in Figure A, the portion of Eps15R which contains the EH domains

shares 70% identity with Eps15 [4]. The homologue of mammalian Eps15 has been identified also in yeast (ede1), *C. elegans* (EHS-1) [3, 19] and in *D. melanogaster* (dEps-15) [20]. In yeast, ede1p is involved in fluid-phase endocytosis and the clathrin-dependent endocytosis; EHS-1 and dEps-15 are expressed mainly in neurons, where they play a role in synaptic vesicle recycling [19, 20], a specialized form of clathrin-dependent endocytosis.

The structure of Eps15 and Eps15R consists of three main regions. The N-terminal portion contains three EH domains, the central region of α-helix forming a coiled-coil region, followed by a C-terminal region containing repeated motifs of the tripeptide sequence DPF (aspartic acid-proline-phenylalanine). In addition, a proline-rich region [1, 4, 21] and two short sequences defined as Ubiquitin-interacting-motif (UIM) domains, which are able to bind monoubiquinated proteins were identified (Refer to Figure D) [22]. The EH domains of Eps15 were shown to interact with NPF-containing proteins such as Epsin, Numb and Hrb. The DPF motifs were found to interact with clathrin assembly adaptor proteins such as AP-2, whereas the coiled-coil region is thought to play a role in homo- or hetero-dimerization. The interactors of Eps15 will be described in detail in the following sections that are divided according to the functions or the processes, in which Eps15 and the interactors are involved in.

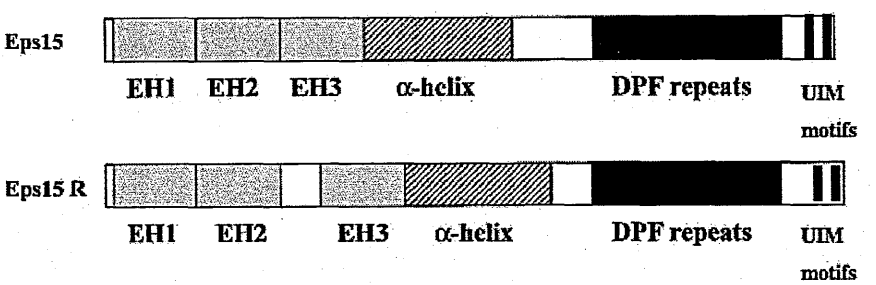
2.1.2 Eps15 and clathrin-dependent endocytosis

The DPF repeats in the C-terminal region of Eps15 were found to be responsible for the interaction with the α-subunit of AP-2 [23, 24], an adaptor complex able to interact directly with clathrin during vesicle formation [25-28]. Immuno-electron microscopy data revealed that Eps15 is localized to the rim of budding clathrin-coated vesicles at

the plasma membrane [29] and then released from the forming vesicle when the clathrin coat is assembled [30].

The importance of the EH domains in the function of Eps15 in the clathrin-coat assembly and the endocytosis was demonstrated by the study of a deletion mutant of Eps15, which lacked the second and the third EH domains. Transient overexpression of this construct was disrupted the localization of Eps15, as well as the distribution of AP-2 and clathrin and Dynamin at the plasma membrane. The cells expressing the mutant Eps15 lacking the EH domains was also shown to have reduced the internalization of transferrin, which is used as a marker for clathrin dependent endocytosis [31]. The complex of Eps15 and AP-2 was also shown to interact with Epsin, an adaptor protein involved in the clathrin-coated assembly [13].

Figure D. The Structure of Eps15 and Eps15R. (Adapted from the figures taken from the articles by Salcini A.E., et al. [11] and Polo S., et al. [22])



In addition to the interaction with the clathrin-assembly adaptors such as AP-2 and Epsin, Eps15 also interacts with dynamin [19, 31, 32], a GTPase that is required for the vesicle fission during endocytosis [33, 34]. Benmerah *et al.* showed that dynamin is no longer localized in puctate pattern in the plasma membrane region, when the dominant negative form of Eps15 is expressed [31]. More recently, the homologues of Eps15 and Dynamin in *C. elegans, ehs-1* and *dyn-1* respectively, were also shown to interact

genetically [19]. In the same study [19], the physical interaction between Eps15 and Dynamin I and II was demonstrated *in vitro*.

2.1.3 Eps15 in synaptic vesicle recycling

Eps15 is enriched at the presynaptic nerve terminals, which is the site of a specialized form of clathrin-dependent endocytosis [13]. Eps15 has been shown to interact with AP180, which is a Vesicle recycling at the synapses uses the molecular mechanisms of the clathrin-dependent endocytosis. Eps15 has been reported to undergo a stimulation-dependent dephosphorylation at nerve terminals [35]. Dephosphorylated Eps15 was shown to bind to the α-adaptin appendage domain with higher affinity, suggesting that the dephosphorylation leads to an increased efficiency of the synaptic vesicles membrane internalization [35]. Furthermore, the study of *ehs-1* (strain *ok146*), the Eps15 mutant *in C. elegans*, showed that the mutants suffered a temperature sensitive locomotion defect, which was attributed to the presynaptic neurotransmission defect [19]. The analysis of dEps15 homologue in *Drosophila* also revealed a function in the nervous system [20].

2.1.4 Eps15 and non-clathrin endocytosis

Eps15 and the aforementioned interactor, Epsin, both contain the Ubiquitin-Interacting Motif (UIM), which binds to ubiquitin moiety. Upon the EGF stimulus, EGFR is monoubiquitinated on multiple sites and the UIM domain of Eps15 was shown to bind to the monoubiquitinated EGFR [36]. The UIM domains of Eps15 participate in the mechanism of coupled-monoubiquitination, as these domains mediate the interaction between the ubiquitinated proteins and Eps15, which is often ubiquitinated itself. The UIM domains of Eps15 are thought to interact with ubiquitinated EGFR, which was implicated in the mechanism by which the dosage of EGF influences the internalization

pathway of EGFR. In the presence of low level of EGF (1.5 ng/ml), EGFR is localized to the clathrin-dependent endocytosis, where EGFR is tyrosine-phosphorylated and is capable of downstream signalling. In comparison, in the presence of high EGF concentration (20 ng/ml), EGFR is ubiquitinated and distributed equally between the clathrin-coated pits and the caveolae [37-40].

2.1.5 Other functions of Eps15

Prior to the screenings carried out by this project, several proteins had been found to interact with the EH domains of Eps15. Numb and Numb-l were identified in a screening of an expression library with the EH domains of Eps15 [11]. Numb was originally identified in *D. melanogaster* [41]. Both Numb and its related protein, Numb-like (Numb-l), are asymmetrically localized and involved in cell-fate determination of progenitor cells during neurogenesis [42] and myogenesis [43]. Numb contains an NPF motif in its C-terminus, through which it binds to the EH domain of Eps15 [11]. In its N-terminus, Numb contains a PTB domain that has been demonstrated to bind to a membrane-associated receptor, Notch [44, 45], which is also involved in the decision of the cell-fate. The interaction between Eps15 and Numb, and its link to Notch via Numb suggests that Eps15 is also implicated in the cell-fate determination during development [46, 47].

The same screening that identified Numb and Numb-l as the interactors of Eps15, also identified Hrb and its related protein Hrb-l as interactors of the EH domains of Eps15 [11]. Hrb is also known as Rev-interacting protein. Rev is an HIV-1 protein, containing a zinc-finger domain in the N-terminal portion and four NPF motifs in its C-terminus [3]. Hrb has been shown to act as a cofactor of Rev, which is involved in the nucleo-cytosolic shuttling of the viral transcripts in HIV-infected cells. Another known

interactor of Eps15, epsin has been reported to undergo nucleo-cytosolic shuttling, together with several other endocytic proteins [48-50].

2.2 Intersectin family

2.2.1 Introduction on the Intersectin family

Two genes (Intersectin 1 and 2) encoding similar proteins were identified in mammals and were found conserved throughout evolution. Clear homologues has been found in lower eukaryotes such as *Drosophila* (*Dap160*) and *C. elegans* (*itsn-1*). In yeast, a putative Intersectin homologue can be identified in *pan1*, which is an EH domain-containing protein, and shares with the Intersectin proteins a role in actin organization. Intersectin was first identified when the cDNA library of *X. laevis* oocytes was screened with an SH3 peptide ligand. The *Xenopus* gene was identified to contain two EH domains in its N-terminus, a central coiled-coil region, and five SH3 domains in the C-terminus [14].

The EH domains of Intersectin bind NPF motif-containing proteins, such as Epsin, Hrb/Hrb-l and Numb [14, 51], which interact also with the EH domains of Eps15 (Refer to Section 2.1.2 and 2.1.5). In addition, a component of recycling vesicles, Secretory Carrier Membrane Protein 1 (SCAMP1), which also contains NPF motifs, was recently discovered to interact with the EH domains of Intersectin [52]. The SH3 domains of Intersectin interact with several proteins, such as the Son-of-Sevenless, Dynamin, and Synaptojanin [14]. When the mouse Intersectins (initially called Ese1 & 2) were identified, the coiled-coil region of the Ese1 was reported to interact with the coiled-coil region of Eps15 by a Yeast Two Hybrid approach [53]. Many interactions mediated by the different domains of Intersectin suggest diversity in their functions. Each

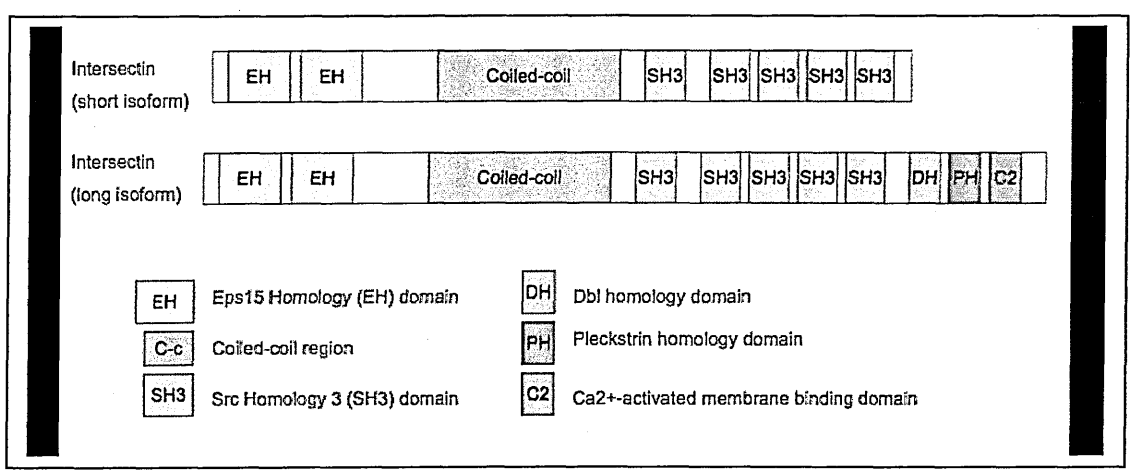
intracellular process, in which Intersectin and its interactors are implicated, will be discussed in later sections.

In addition, the mammalian Intersectin proteins contain isoforms with extra C-terminal regions due to alternative splicings. The extended isoforms, which are more neuronal specific, contain a Dbl homology (DH) domain, a Pleckstrin homology (PH) domain and a C2 domains [53-56] (Figure E). The DH and PH domains specific to the mammalian long isoforms have been shown to play a role in actin cytoskeleton organization, which will be discussed later, in Section 2.2.4.

2.2.2 Intersectin and clathrin-dependent endocytosis

The interactions with endocytic proteins, such as Dynamin, Eps15, epsin and Synaptojanin [14], strongly implicate Intersectin in endocytosis. At first, the role of Intersectin was suggested to take place mainly in clathrin-dependent endocytosis [14, 56]. Indeed, overexpression of wild type Intersectin proteins was sufficient to reduce the internalization of transferrin and EGF. Accordingly, mammalian Intersectins were found targeted to clathrin-coated pits and the importance of the interactions mediated by the SH3 domains of Intersectin in clathrin-coated vesicle formation has also been shown [57].

Figure E. The structures of Intersectin long and short isoforms. (Adapted from the review by Santolini et al. [3])



2.2.3 Intersectin in synaptic vesicle recycling

The function of Intersectin at the level of presynaptic termini is better appreciated in lower organisms such as *Drosophila* and *C. elegans*. *Drosophila* homologue, Dap160, was identified as a membrane-associated neuronal protein that interacts with dynamin [58]. Further studies on Dap160 revealed that it acts as a scaffolding protein required for synaptic development and synaptic vesicle retrieval [59, 60]. A recent report demonstrated that Dap160 plays its role in scaffolding structure together with Eps-15 at the site of synaptic vesicle endocytosis [61]. Furthermore, the Intersectin homologue studied in lamprey eel showed that it negatively regulates the level of dynamin redistributed at the periactive zone, and that Intersectin may play a role in the fission of vesicles [62].

A recent study of Intersectin in *C. elegans* also demonstrated ITSN-1 as a neuronal protein, with a role in synaptic transmission. In addition, the study on *itsn-1* has shed light for the first time on a possible antagonistic relationship between *itsn-1* and *ehs-1* on the dynamin-regulated pathways [63]. As Intersectin, Eps15 and Dynamin interact with each other to orchestrate the clathrin-dependent endocytosis, the antagonism uncovered by the genetic studies in *C. elegans* suggest a complex mechanism by which the endocytic process is controlled by these proteins.

2.2.4 Intersectin and actin cytoskeleton organization

The DH domain of the Intersectin long isoform has been found to specifically act as a guanine nucleotide exchange factor for Cdc42, but not for Rac1 or RhoA [64, 65]. Intersectin was shown to interact with Wiskott Alrich Syndrome Protein (WASP) or Neural-WASP (N-WASP), which participate in the Arp2/3 complex mediated-actin polymerization [64, 66]. Recently, Numb was also identified as an interactor of Intersectin, and the presence of Numb was shown to enhance the Cdc42 GEF activity of

Intersectin [51]. Numb, a protein that determines cell-fate is involved in the neurite outgrowth during the early stages of the neuronal development. Numb plays a role in the formation of dendritic spines in the later stages of the neuronal development. Numb is thought to activate the GEF activity of Intersectin to promote the actin cytoskeleton re-arrangement mediated by the Cdc42, which is required during the morphogenesis of the dendritic spines. In addition, the PH domain of the Intersectin was also shown to act together with the DH domain in the regulation of the GTPase activity [67].

2.2.5 Intersectin and exocytosis

In agreement with the ability of Intersectin-1 long isoform to activate Cdc42, Intersectin was found to play a role in the Cdc42-mediated exocytic pathways [68]. In further support of its role in exocytosis, rat Intersectins EHSH1 and EHSH2 were shown to interact with an exocytic protein, SNAP-25 (Synaptosomal associated protein of 25kD) in yeast two hybrid screens [54]. SNAP-25 was originally identified as a synaptosomal protein [69]. It was later discovered as a protein targeted by the Botulinum neurotoxin type-B, indicating that it is a component of the core complex in the docking of vesicles to the plasma membrane during exocytosis [70].

Another interactor of Intersectin involved in exocytosis, is SCAMP1, a secretory protein also implicated in endocytosis, that binds to Intersectin's EH domains [52]. The fact that Intersectin-1L plays a role in the actin cytoskeleton organization required during exocytosis implies that Intersectin is an adaptor protein, which coordinates the membrane trafficking of both endocytosis and exocytosis.

2.2.6 Intersectin and mitogenic signalling

In addition to interactions with endocytic proteins such as Dynamin, the SH3 domains

of Intersectin also mediate the interactions with several proteins that regulate cellular signaling pathways. For example, Intersectin forms complexes with the guanine nucleotide exchange factor, Sos, to stimulate Ras activation specifically on endomembrane compartments [71, 72]. More recently, it was shown that Intersectin associates with the Cbl, an E3 ligase of EGFR, and this association promotes the receptor ubiquitylation and degradation [73].

In addition, Intersectin expression was sufficient to induce an oncogenic transformation of rodent fibroblasts. The overexpression of the tandem EH domains of Intersectin was found to be sufficient to activate the Elk-1 transcription factor in a MAPK-independent manner [74]. Furthermore, Intersectin cooperated with epidermal growth factor to potentiate Elk-1 activation.

The function of Intersectin-L in cell growth signalling was further investigated when the substrate of its GEF activity, Cdc42, was implicated in cell transformation. It was reported that the mutated Cdc42 (Cdc42 (F28L)), which is constitutively active, activated c-Jun Kinase (JNK) and induced transformation of the cultured fibroblast cells [75]. Various portions of Intersectin-L are able to transform cultured cells and mutational studies on the DH and the SH3 domains indicated that both regions are required for the activation of JNK and PI3K (Phophatidyl-inositol-3 Kinase) [76]. The study suggested a complex interplay between the activation of the pathways of Ras and Cdc42 in the cell proliferation control. Together, these data suggest that Intersectin links endocytosis with regulation of pathways important for cell growth and differentiation.

2.2.7 Other functions of Intersectin

Recently, Intersectin was found localized at the caveolar-neck in endothelial cells. The study demonstrated that Intersectin is required for an efficient fission of caveolae, suggesting that, through its interaction with dynamin, it also plays a role in clathrin-independent but dynamin-dependent internalization [77].

As mentioned earlier in the introduction section 2.2.1, the EH domain of the *Xenopus* Intersectin was shown to interact with Hrb and Hrbl, The interactions with Hrb/Hrb-l, which were previously described as interactors of the EH domain of Eps15, suggesting a possible role for Intersectin in nucleo-cytosolic shuttling [14].

Intersectin was also found to play a role in cell survival. Decreasing Intersectin expression dramatically increased apoptosis in both neuroblastoma cells and primary cortical neurons [78] and in endothelial cells [79]. A yeast two-hybrid analysis identified class II phosphoinositide 3'-kinase C2beta (PI3K-C2beta) as an Intersectin-binding protein, suggesting that Intersectin may regulate a PI3K-C2beta-AKT survival pathway.

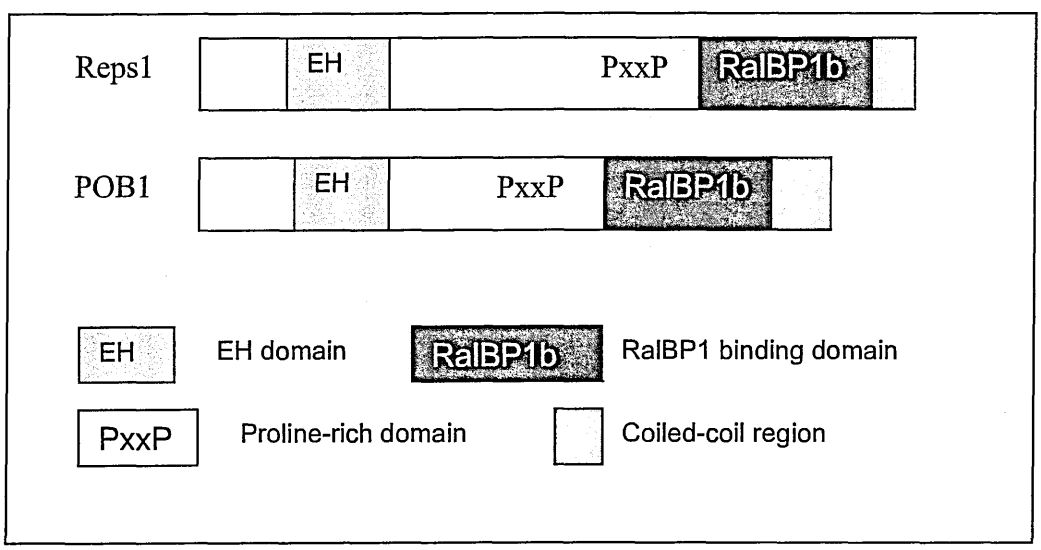
2.3 Reps family

2.3.1 Introduction on the Reps family

The Reps family is composed of two closely related proteins, Reps1 and Reps2/POB1 (Figure F). Reps1 was first identified as a human gene, encoding for an interactor of RalBP1, (RalBP1-Eps homology domain protein 1) [80], but homologous proteins have been identified in *Drosophila* and *C. elegans*. Reps family proteins contain one EH domain, a proline-rich domain, and the RalBP1 binding domain. The EH domain of

POB1 interacts with an EH protein, Eps15, and an endocytic adaptor protein, Epsin, that also interacts with Eps15 and Intersectin (See Sections 2.1.2 and 2.2.2). The interaction with another EH protein mediated by an EH domain has so far been observed only for POB1, and the nature of this interaction will be discussed in the following section 2.3.2. In addition to endocytic interactors, Reps/POB1 family is likely to participate in various processes that involve RalBP1. RalBP1 binds specifically to GTP-bound form of Ral proteins, which are a family of multifunctional small GTPases in mammalian cells, regulating processes as diverse as membrane transport, apoptosis, transcription, cell migration, cell proliferation and oncogenesis [81]. In agreement with the role of RalBP1 in oncogenesis, POB1 expression was found to be downregulated during the progression of prostate cancer. It was suggested that POB1 is involved in growth factor signalling through Ral, and the decreased level of POB1 leads to the loss of control of cell proliferation in tumorigenic cells [82].

Figure F. The structures of Reps1 and POB1. (Taken from the review by Santolini et al. [3])



2.3.2 Reps1/POB1 in endocytosis

Both Reps1 and Reps2/POB1 are tyrosine-phosphorylated upon EGF stimulation, leading to the interaction between these proteins and the EGFR [80, 83]. Moreover, overexpression of the EH domain of POB1 leads to a decreased internalization of EGFR and Insulin receptor, suggesting that POB1 regulates receptor internalization and that the EH domain is important for this function. Pull-down experiments using a GST-fusion protein of the EH domain of POB1 from the bovine brain membrane extracts identified Eps15 and Epsin [15]. Interestingly, in the report by Nakashima *et al,* the binding of the EH domain of POB1 to Eps15 C-terminal region suggested that the POB1 EH domain is capable of binding to a region not containing NPF motifs [15]. Indeed, the EH domain of POB1 was shown to recognize the DPF motifs, many of which are present in the C-terminal region of Eps15 [84].

During the mitotic phase, Epsin, POB1 and Eps15 are serine- phosphorylated and this phosphorylation inhibits the complex formation, suggesting that the complex is involved in the specific control of endocytosis during the mitosis [85].

2.3.3 Reps1/POB1 and Recycling

Reps1 also plays a role in endosomal recycling, as suggested by its interaction with a member of the Rab11-binding protein family, Rab11-FIP2 [86]. Rab proteins such as Rab4 and Rab11, orchestrate the protein sorting into the endocytic recycling compartments (ERC) at the early endosomes, and out of the ERC at the plasma membrane. Rab11-FIP2 is one of the Rab11-binding effector proteins that function with the Rab proteins in the endosomal recycling. It also interacts with Myosin Vb [87], and it was shown to associate with the endosomal membrane [88]. Interestingly, Rab11-FIP2 also interacts with another EH protein family, the EHD family of proteins that are

mainly implicated in the endocytic recycling and endosomal transport [89]. This will be discussed later in the section on the EHD family, in Section 2.5.2.

2.3.4 Reps1/POB1 and cell migration

An additional function of POB1 is indicated by the interaction between the third proline-rich region in the C-terminal domain to the SH3 domain of ASAP1, a mouse homologue of a human protein PAG2, a paxillin-associated protein, which inhibits the fibronectin-dependent migration and paxillin recruitment to focal contacts. The study suggested a role of POB1 in cell migration through its interaction with PAG2 [90].

2.4 EHD family

2.4.1 Introduction on the EHD family

The main feature of the EHD family of proteins is that they all contain a C-terminal EH domain rather than an N-terminal one, like the Eps15 and Intersectin families (Figure B). The EHD family has been found to be conserved in nematode, fly, mouse and human, and one EHD1 gene in the human malaria parasite, *Plasmodium falciparum* has also been reported [91]. The EHD1 gene has been studied as a candidate gene found in the locus of two different pathologies, the Multiple Endocrine Neoplasia 1 (MEN1) [92] and Bardet-Biedl Syndrome (BBS1) [93, 94].

Following the identification of EHD1, three other members of the mammalian EHD family have been identified, namely EHD2, EHD3 and EHD4 [95]. As indicated in Figure B, EHD proteins also contain a coiled-coil domain in the central region, as well as a nucleotide-binding region near the N-terminus [96]. Numerous studies have indicated that the nucleotide-binding is a crucial mechanism in the function of the EHD proteins. For example, the homo-/hetero-oligomerization of the EHD proteins that is

mediated by the coiled-coil region was shown to be an ATP-dependent process [97, 98]. Similarly, the association with membrane structure was shown to be nucleotide-dependent [99]. This will be discussed in the section 2.4.4.

2.4.2 EHD proteins and recycling

The EHDs share many common features with the dynamin superfamily, such as a low affinity for nucleotides, ability to tubulate liposomes *in vitro*, oligomerization around lipid tubules in ring-like structures and stimulated nucleotide hydrolysis in response to lipid binding [100]. As previously mentioned in the section 2.1.2, dynamin is a GTPase that allows the fission of the neck of vesicles at the membrane. Despite the similarities between EHD proteins and dynamin, numerous studies on EHD proteins have revealed that they are involved in the recycling of endocytic compartments rather than the fission of vesicles during endocytosis.

An *in vivo* assay of yolk-protein uptake in *C. elegans* demonstrated that the *C. elegans* homologue of EHD1, RME-1, is involved in the recycling of vitellogenin receptors internalized by clathrin-dependent endocytosis [99]. A recent report in *C. elegans* showed that RME-1 interacts with ALX-1, a member of Alix/Bro1p family that interacts with the endosomal ESCRT complex-I and -III. The interaction between RME-1 and ALX-1 is required for the recycling from the endosomes to the plasma membrane [101].

Studies on mammalian EHD proteins also revealed that they play a role in the trafficking of endocytic recycling compartments (ERC). The receptors that have been shown to be recycled to the plasma membrane in a EHD1-dependent manner are: transferrin receptors [102], cystic fibrosis transmembrane conductance regulator [103],

the insulin-regulated GLUT4 [104, 105], and AMPA-type glutamate receptors at the post-synaptic membranes [106].

To date, EHD1, EHD2 and EHD3 were shown to interact with the Rab-binding proteins, providing a physical link to the Rab family of proteins that coordinate the sorting of proteins in the ERC. As briefly mentioned in the section 2.3.3, EHD1 and EHD3 interact with an effector protein of Rab11, Rab11-FIP2 [89]. In addition to Rab11-FIP2, the EH domains of the mammalian EHD1 and EHD2 interact with the NPF motifs of Rabenosyn-5 [107], which forms a complex with Rab 4 or Rab5 GTPase proteins and hVPS45 [108, 109].

Syndapins, which are interactors of Dynamin and N-WASP, and play a role in endocytosis, vesicle recycling, and actin organization [110, 111], were also shown to interact with the EHD proteins. The EH domains of four EHD proteins (EHD1, EHD3, EHD4 and *C. elegans* RME-1) interact with the NPF motifs present in Syndapin I and Syndapin II [112].

Furthermore, EHD1 was found localized on tubular structures that associate with ADP-ribosylation factor 6 (Arf6), which mediates the internalization of Major Histocompatibility Complex 1 (MHC-I) via lipid raft [98]. Other EHD proteins were also found to play a role in the Arf-6-mediated endosomal transport of non-clathrin endocytic compartments. For example, the EHD protein homologue in *Drosophila,* dEHD, and the mammalian EHD4 were both shown to interact with Numb [113], an NPF-containing protein that interacts also with Eps15 and Intersectin (Refer to the Sections 2.1.5 and 2.2.4). The interaction between EHD4 or dEHD and the NPF-motifs in the C-terminal of Numb was shown to be induced at sites of Arf6-mediated endocytic

recycling [113]. Moreover, EHD4 was shown to co-localize with the markers of early endosomes, such as Rab5 and early embryonic antigen 1 (EEA1), and the endosomes in which EHD4 was localized were Arf6-containing endosomes [113]. Furthermore, a very recent report on EHD1 and EHD4 implicates both EHD proteins in the early endosomal transport of the clathrin-independent endocytic compartments [114].

2.4.3 EHD proteins and vesicle fusion

EHD1 interacts with a SNARE protein SNAP-29 [115, 116]. SNAP-29 and EHD1 were found in a complex with Insulin-like Growth Factor 1 Receptor (IGFR) and were thus implicated in the regulation of the IGF-signalling pathway [115]. However, SNAP-29 has been reported as a general SNARE protein implicated in synaptic transmission [117, 118] and it was shown to inhibit the disassembly of SNARE complex [119]. Taking into consideration that SNAP-29 shares 39% sequence similarity to neuronal specific SNARE protein SNAP-25, and 35% similarity to ubiquitous SNAP, SNAP-23 [115], the interaction between SNAP-29 and EHD1 may participate in the vesicle fusion events both at the synapse and at the endocytic sites in non-neuronal cells.

2.4.4. EHD proteins and membrane remodelling

Recently, EHD2 was shown to interact with membrane phospholipids, implicating the EHD proteins in the induction of membrane curvature [100, 120]. As mentioned earlier briefly in the introduction, the nucleotide-binding domain of EHD is required for the oligomerization of the proteins. In turn, the oligomerization of EHD proteins uncovers a highly curved membrane-binding domain. In agreement with this model, the mouse and *C. elegans* EHD1 were shown to bind and hydrolyse ATP, and the interaction with ATP was shown to be a prerequisite for EHD to bind to the endosomal membrane [121].

The interactions between EHD1 or EHD3 and the NPF motifs of endosomal recycling proteins such as Rab11-FIP2, Rabenosyn-5 and Syndapins (See Section 2.4.2), were shown to be reduced when the nucleotide-binding to the p-loop of the EHD proteins was impaired [89]. A study with the mutated oligomerization domain of EHD1 and EHD3 demonstrated that oligomerization is also required for the interaction between the EH domain of the EHD proteins and NPF motifs [89]. The nucleotide-binding and the oligomerization of EHD proteins are required for the EHD binding to the membrane. These data together suggest that the EHD proteins are localized at the endosomal membranes when they interact with NPF-containing proteins, thereby recruiting the interactors to the endosomal or synaptic vesicle recycling compartments.

2.4.5 EHD proteins and actin cytoskeleton

The interaction of EHD proteins with Syndapin I and II, which were described in the previous section 2.4.2, implicates the EHD proteins in the actin cytoskeleton organization. Syndapins are thought to act as the link between the endocytosis and actin remodelling [111]. The EH domain of EHD2 was shown to interact with the NPF motif of EHBP1 (EHD2-Binding Protein 1) [122]. EHD2 and EHBP1 were shown to function in the endocytosis of receptors such as GLUT4 and transferrin in adipocytes. More interestingly, EHD2 and EHBP1 were demonstrated to regulate actin cytoskeleton organization through an acidic region and a CH (Calponin Homology) domain, respectively. The acidic region adjacent to the EH domain in EHD2 has been suggested to act as an Arp2/3-binding motif, emphasizing the link between the endocytosis and the actin rearrangement by the complex of EHD2 and EHBP1. Syndapins, which have been described previously in this section as binding partners of EHD proteins [112], were suggested as the coordinators of the machinery for the actin nucleation events at various stages of endocytosis [123].

2.4.6 EHD and endosomal signalling

An implication of EHD proteins in the endosomal signalling comes from the studies on an EHD family protein, Pincher, which is most similar to the EHD5. Pincher acts in the Nerve Growth Factor (NGF)-induced pinocytosis of TrkA receptor, and it is involved in retrograde signalling of Trk-signalling endosomes [124]. Retrograde transport of the neurotrophic signal, such as NGF activation of TrkA, is a critical process in promoting the cell survival of neurons. In neurons, receptors such as TrkA, are activated by neutrophins at the axonal terminal. The activated receptors are internalized and are transported in endosomes rapidly to the cell bodies, utilizing the transport through the cytoplasm that is motored by dynein [125].

As the receptors internalized via clathrin-dependent pathway are usually destined for degradation or recycling to the plasma membrane, the internalization of the neurotrophin receptors is thought to occur via clathrin-independent pathways, in order to transport the signal. Neutrophin signalling in neurons is a critical process for maintaining the cell survival, and the failure of such process is thought to be the cause of the loss of neurons observed in the patients with neurodegenerative diseases. The implication of the EH proteins and their interactors in the neurodegenerative diseases will be discussed at length in the Discussion (Section 16.2).

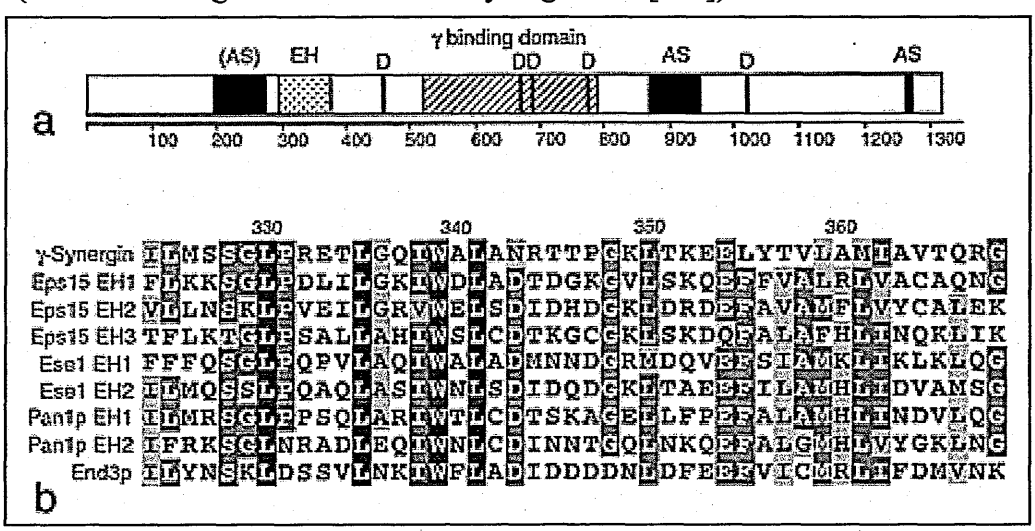
2.5 γ-Synergin

γ-synergin is an EH domain-containing protein, which was found as an interactor of γ-adaptin, a subunit of the adaptor protein complex-1 (AP-1). AP-1 is a complex of adaptor proteins that orchestrates the clathrin-coat assembly at the Trans Golgi Network

(TGN) [126]. γ-synergin was found associated with AP-1 in the cytosol, on the membranes of the TGN, and in the clathrin-coated vesicles [127]. The structure of γ-synergin consists of an EH domain and the γ-binding domain in the central region of the protein, as illustrated in Figure G. The alternative splice sites (AS) and the copies of the DDFD/EXF motifs (D), will not be discussed here as they are not crucial for the function as an EH protein.

The EH domain of γ-synergin interacts with an NPF-containing protein SCAMP1 [128], which was initially implicated in exocytosis. It also interacts with Intersectin, as described in the section 2.2.6. The transmembrane protein SCAMP1 is suggested to act as the docking site for the cytosolic γ-synergin at the membrane of the TGN.

Figure G. The structure of gamma-synergin
(a) the alignment of the EH domain of γ-synergin with the other EH domains (b).
(Taken from Figure 3 of the article by Page et al. [127])



In addition, γ-synergin was recently found in a stable complex with Aftiphilin and p200 that assists the function of AP-1 [129]. The targeting of γ-synergin to the TGN is thought to be mediated via the signal sequence found in the γ-adaptin of AP-1 [130]. γ-synergin was also shown to interact with the ears of Golgi-localized, γ-ear containing,

ARF-binding proteins (GGAs), a family of monomeric adaptor proteins that contain the C-terminal regions homologous to γ-adaptin [131-134].

In comparison to the other EH proteins, the various interactors of γ-synergin that have been identified to date implicated its function specific to the assembly of the clathrin-coated vesicles at the TGN, a crucial process in protein secretion. The specific function may rise from the fact that γ-synergin is an EH protein specific to mammals, although putative homologues have been identified in the other species. characterized to date. Moreover, the protein structure of γ-synergin consists of only two domains, the EH domains and the γ-binding domain, whereas the other EH proteins contain binding sites for diverse proteins. The predicted *C. elegans* homologue of γ-synergin encoded by the gene R10E11.6 has remained an uncharacterized gene to date. Y2H screenings in this study searched the *C. elegans* genome for any interactors of the putative EH domain of R10E11.6.

3. Experimental Approach

3.1 Introduction

The current knowledge of the EH domain-containing proteins (EH proteins) and their interactors illustrates that the proteins in the EH network are involved not only in endocytosis, but in various intracellular processes. In order to better understand the extent of the network of different intracellular signalling pathways mediated by the EH proteins, this project was designed as a genome-wide screening of interactors of EH proteins in a model system, *C. elegans*.

The nematode was chosen for several reasons. For example, the *C. elegans* genome contains single non-redundant homologues of each four major EH protein families. *C. elegans* has also been established as an ideal organism for *in vivo* genetic studies, which were carried out subsequent to the Y2H screenings. The advantages of using *C. elegans* as a model system will be explored in detail in the following section.

The aim of this study was to identify all putative interactors of EH proteins in the whole genome of the model system. Therefore, the EH domains of each EH protein present in the *C. elegans* genome were cloned as separate baits for Y2H screenings against a *C. elegans* cDNA library.

3.2 Yeast Two Hybrid Screenings

3.2.1 Introduction to the Y2H system

Y2H system is a widely used technique to test the interaction between two moieties. It utilizes the yeast genetic system to select the cDNAs ("preys") that encode the proteins that bind to the target, or the "bait" proteins. The bait is usually cloned in-frame with the DNA binding domain (BD) whereas the prey cDNA is cloned in-frame with the transcription activation domain (AD), to express two recombinant proteins in yeast (Figure H). When the prey that is expressed in-frame with the AD interacts with the bait protein, the complex of the hybrid proteins form a functional transcriptional activator.

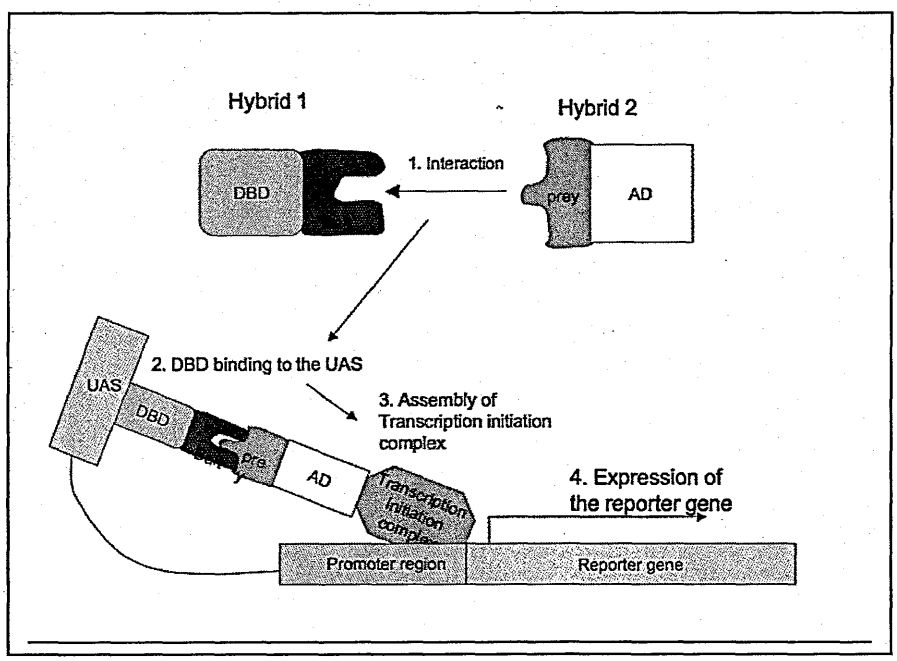
In this study, the hybrid proteins with the GAL4-DBD and GAL4-AD were used. The genome of the strain *Mav203* of *S. cerevisiae*, which was used in this study, contains three reporter genes with the GAL4-promoter region. Transcription activation of the reporter genes such as *URA3* and *HIS3* are monitored by growth in the selective medium lacking uracil and/or histidine, In comparison, the activation of the third reporter gene, *LacZ*, is detected by an assay called the X-gal assay, in which the β-galactosidase encoded by *LacZ* catalyses the reaction of X-gal, resulting in the colour change from white to blue yeast cells.

3.2.1 Advantages of the Y2H system

The strongest advantage of the Y2H system is that the bait can be designed to contain a limited fragment of a protein, such as a specific domain, as seen in this study. A bait engineered to contain mutations of interest can also be tested in comparison to the interaction mediated by the wild type bait. For example, Naslavsky *et al.* mutated the

critical residue of the nucleotide-binding domain of EHD1 and EHD3 (G65R) and tested the oligomerization of EHD1 and EHD3 by Y2H [89].

Figure H. Yeast Two Hybrid system for detecting protein interactions.
(1) The interaction surfaces on bait in the recombinant protein 1 (Hybrid 1) and the prey in the recombinant protein 2 (Hybrid 2) interact to form a complex. (2) The DBD binds to the upstream activating sequence (UAS) of the reporter gene. (3) The AD stimulates the assembly of the transcription initiation complex at the promoter region of the reporter gene. (4) The transcription of the reporter gene is activated.



The technique of cDNA library preparation has enabled the application of the Y2H system a genome-wide screenings. Furthermore, the possibility to use the cDNA library as the prey allows the identification and the cloning of the putative interactor directly from the plasmid purified. The use of yeast system also allows an easy handling and maintains a low cost, even with large-scale transformations, such as that needed in screenings. For the analysis of two candidate interactors, only a small amount of DNA, 1ng of each recombinant construct, is sufficient to obtain several clones to be tested for the expression of the reporter genes, providing a quick and easy method.

In comparison to Y2H, interactors identified by other biochemical approaches, such as co-immunoprecipitation coupled with the protein identification by mass spectrometry, may be biased, depending on the sample preparation. In the case of co-immunoprecipitation, the representation of proteins found in the sample can be affected by several factors. For example, the protein population purified from cell lysates may vary due to the endogenous level of expression, the conditions of cell lysis buffer and the sensitivity of the instrument used for the identification of the protein mixture. By expressing putative interactors in yeast, even weak or transient interactions can be detected by Y2H system.

3.1.2 Disadvantages of Y2H system

On the other hand, Y2H system has a major disadvantage that interactions shown by the system may be false positives. When the hybrid proteins are expressed in yeast, they are confined in a small space, enabling proteins to interact even though they are often not localized together in a physiological condition. For example, a nuclear protein may interact with a plasma membrane protein, simply because they contain sequences that are "sticky". The intracellular localization and the expression pattern in tissues should be considered when analyzing the interactions identified by the Y2H system.

Therefore, different methods to detect protein-protein interactions should be used to complement each other. Y2H system is more suitable for a large-scale screening to fish out putative interactors from a mixed population of proteins expressed by cDNA library. The interactions identified in the Y2H should be validated by *in vitro* pull-down assays, or by a method that better reflects the endogenous interactions, such as co-immunoprecipitation.

3.2 *C. elegans* as a model system

C. elegans is a favourite model organism for genetic studies, such as the one carried out in this project. The main benefit of *C. elegans* for this study is that the EH protein families were represented by one non-redundant homologue in its genome. For instance, *C. elegans* contain one Eps15 homologue (EHS-1) and one Intersectin homologue (ITSN-1), whereas *S. cerevisiae* EH proteins such as Pan1p, End3p and Ede1p do not contain the other non-EH domains to match as the clear homologues of the mammalian Eps15 and Intersectin. Instead, each mammalian EH protein family contains different members, as described for each family in the Section 2 of the introduction.

C. elegans was an appropriate model system to study the EH network also for the fact that there are mutant strains available for *ehs-1* [19], *itsn-1* [63], and *rme-1* [99], and a recently discovered *reps-1*. The reported phenotypes of the EH mutants allowed the monitoring of genetic interactions with the putative interactors identified in the Y2H system.

In addition to the advantages specific to this project in using *C. elegans*, there are numerous advantages of *C. elegans* as a model system *per se*. First of all, *C. elegans* provides several practical advantages in laboratories. They are small (approximately 1mm in length) and easy to cultivate, and *E. coli*, on which they feed, and the medium required for cultivation, are cheap. Moreover, the organism is transparent, allowing the cells of interest to be studied under the microscope *in vivo*..

The short life cycle is another factor that facilitates the cultivation of *C. elegans* in laboratory. *C. elegans* reach adulthood at an optimum temperature of 25°C in 3 days. Cultures of *C. elegans* can be maintained at a temperature from the range of 12°C to

25°C. This allows an additional benefit as the growth rate of the cultured nematodes can be increased or decreased depending on the temperature. The oocytes are self-fertilized in the spermateca in the hermaphrodite nematodes, the embryos develop in eggs until the eggs are laid. Worms of larval stage 1 (L1) hatch from the eggs, and develop through various larval stages from the L1 to L2, to L3 and to L4, until the worms reach the adulthood. Hermaphrodites can produce offspring also by mating with males, which are formed by stress-induced mutations. Therefore, a cultured strain of *C. elegans* can be induced to form males, which can then be used as means of crossing strains.

The ability to synchronize the growth of *C. elegans* was another useful characteristic, in the genetic studies carried out during this project. In the experiments that monitored the genetic interaction in this study required hundreds of worms to have reached the same developmental stage. By starving the L1 larvae that hatched from the eggs isolated from the hermaphrodite mothers and leaving the synchronized L1s to grow at a stable temperature provided young adult worms on the day of the experiment.

The vast collection of data already available from the past studies provides another advantage for the *C. elegans* community. The genome of *C. elegans* has been completely sequenced and the information is publicly available through a database called the Wormbase, accessed via the Internet at http://www.wormbase.org. The database contains not only sequence information, but also experimental data, such as the phenotypes observed of mutants or of knock-down animals during the RNA*i* screenings of the genome. The expression of the protein can be found as well, where available, and the alignments of sequences with the genes of other species can also be found. In addition, experimental reagents are also readily shared amongst the *C. elegans* community. For example, reagents such as mutants, and the Expression Sequenced Tags (ESTs) are

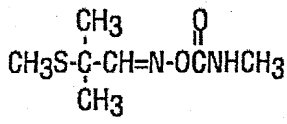
available through various laboratories, such as the *C. elegans* Gene Knockout Consortium, the National Bioresource Project-Japan and the Nematode Expression Pattern Database in the National Institute of Genetics in Japan. Furthermore, the genetic studies by *in vivo* RNA*i* by feeding in the strains of the EH mutants were carried out using many of the *C. elegans* RNA*i* library, which contains the bacterial strains expressing the double-stranded RNA specific for 87% of the whole genome [135].

3.2.2. Studies of Genetic interactions using aldicarb hyper/hyposensitive mutants of *C. elegans*

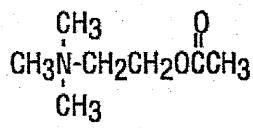
The protein interactions identified in the Y2H screenings in this study were first studied by in vitro pull-down assays for validation of the Y2H results. As a further step of validation, the genes encoding the EH interactors identified by the Y2H screenings were examined whether they genetically interact with the genes encoding the four EH proteins. However, it must be noted that the genetic interactions give an indication that the genes are involved in the same signalling pathways, do not validate the physical interaction between two proteins, such as that shown by the Y2H system or by the in vitro pull-down assays. The genes encoding the EH interactors were knocked-down by RNA*i* by feeding in the mutants of four EH genes, namely *itsn-1*, *ehs-1*, *reps-1* and *rme-1*.

The mutants of two EH genes, *itsn-1* and *ehs-1* both display phenotypes different to that of the wild type N2 Bristol strain, when treated with a compound called aldicarb. The deletion mutant of *ehs-1* (*ok146*) has been reported to be aldicarb resistant [19], whereas *itsn-1 (ok268)* is aldicarb hypersensitive [63].

Aldicarb, a compound commonly used as a pesticide is a competitive inhibitor of acetylcholine esterase. The structure of aldicarb mimics that of acetylcholine, as shown in the illustration below [136]:



aldicarb



acetylcholine:

Inhibition of acetylcholine esterase results in the accumulation of acetylcholine in the synaptic cleft. At the level of a whole organism, for example in *C. elegans*, the accumulation of acetylcholine results in a paralysis that can be monitored by the insensitivity to touch stimuli. Therefore, in the genetic background of the *ehs-1* mutant strain, a higher concentration of aldicarb, or a longer incubation time than that for the wild type, is required to reach the level of acetylcholine in the synaptic cleft that manifests in paralysis. In comparison, a lower concentration or a shorter incubation time is sufficient for *itsn-1* worms to stop responding to the touch stimuli than that for the wild type.

The mutant strain of *rme-1* (*b1045*) was characterized to have a defect in the endocytosis of yolk protein vitellogenin YP170 in the oocytes, as well as a reduced brood size, but no phenotype related to aldicarb treatment has been reported [99]. Therefore, the response to aldicarb by the *rme-1* mutant and the recently found *reps-1* mutant, which is yet uncharacterized, were also studied in this project.

4. Summary of the Introduction

The EH domain, which has been shown to interact with NPF motif-containing proteins, was found conserved through evolution. The EH domain-containing proteins that are found across species are Eps15 family, Intersectin family, EHD family, Reps/POB family, and γ-synergin. Each family of the EH proteins interact with a number of interactors, through which they are involved in diverse intracellular trafficking, such as clathrin-dependent and -independent endocytosis, endosomal recycling, vesicle assembly at the TGN, and nucleo-cytosolic shuttling, Other intracellular processes such as actin cytoskeleton organization, cell fate determination and mitogenic signalling are also found under the EH network.

To date, the extensive picture of the EH network is made up of different studies, mainly overexpression studies in mammalian cell lines, that have shown the functions of the EH proteins or that of the EH interactors. This study aimed to dissect the EH network in *C. elegans*, by identifying the interactors of the EH domains and testing the biological relevance of the interactions in a whole organism. Furthermore, *C. elegans* give us the unique possibility to analyse the impact of the EH network in complex circuits such as synaptic transmission. The interactors identified in this study illustrate a network of proteins that span from the plasma membrane to the nucleus, participating in diverse trafficking and signalling events in non-polarized cells and polarized cells such as neurons.

II. MATERIALS AND METHODS

5. Materials

5.1 Medium for *Escherichia. Coli*

5.1.1 SOC medium

20 g of tryptone, 5 g of yeast extract, and 0.5 g of NaCl was dissolved into 950 ml of dH_2O. 10 ml of a 250 mM KCl was added and the pH was adjusted to pH 7.0 with 5N NaOH. The total volume of the solution was adjusted to 1 L with distilled water (dH_2O) and the solution was sterilized by autoclaving. After the autoclavation, 5ml of sterile 2M $MgCl_2$ and 20 ml of sterile 1M glucose were added.

5.1.2 LB medium (Luria Broth)

10 g of tryptone, 5 g of yeast extract, 10 g NaCl was added to 950 ml of dH_2O and the pH was adjusted to pH 7.0 with 5N NaCl. The total volume was adjusted to 1 L with dH_2O. The medium was sterilized by autoclaving. The antibiotics, Ampicillin or Kanamycin, were added after the autoclaving to the final concentration of 50μg/ml to make a selective medium appropriate for the strain of *E. Coli* used. 15 g/L of Bactoagar was added and sterilized for preparations of solid LB medium

5.2 Medium and specific solutions for *Saccharomyces Cerevisiae*

5.2.1 YPD medium

10 g of BactoYeast extract, 20 g of BactoPeptone and 20 g of dextrose were dissolved in 1 L of dH_2O. The medium was autoclaved for sterilization.

5.2.2 YPAD solid medium

20 g of bactoagar was added to YPD medium and was sterilized by autoclaving.

0.02 mg of adenine sulphate (Sigma-Aldrich) was added immediately after autoclaving. The medium was poured into plates and was left to solidify at room temperature. The plates were stored at room temperature.

5.2.3 Synthetic Complete (SC) medium

5.2.3.1 "Drop-out" mix of Amino Acids

3 g of each essential amino acids (purchased from Sigma-Aldrich) except for Leucine, Tryptophan, Histidine and Uracil were mixed. The container was wrapped with an aluminium foil to avoid damage by light, and was stored at room temperature.

5.2.3.2 SC medium (pH 5.9)

10 g of ammonium sulphate, 3.4 g of yeast nitrogen base (without amino acids) and 2.7 g of amino acid "drop-out" mix were dissolved in dH_2O to a final volume of 1 L. The pH was adjusted to pH 5.9 with 10N NaOH, and the medium was sterilized by autoclaving. After the autoclaving, 80 ml of sterile 40% glucose was added.

Additional amino acids namely Uracil, Histidine, Leucine and Tryptophan, were dissolved in dH_2O and sterilized by filtration through 0.22 μm filters and were added as necessary according to Table A shown below. 20 g of bactoagar per litre of SC medium was added to make a solid medium. The solid medium was stored at room temperature.

Table A: Composition of the amino acids added to the selective SC medium

Amino Acid (AA)	Molarity (mM)	Volume per L of medium (ml)	Final Conc. of AA (mM)
Uracil	10	16	0.16
Histidine	100	8	0.8
Leucine	100	8	0.8
Tryptophan	40	8	0.32

5.2.3.3 10NNaOH

40 g of NaOH was dissolved in a total of 80 ml of dH_2O by stirring continuously. The total volume was adjusted to 100 ml with dH_2O.5.2.4 Solutions for yeast transformation

5.2.4.1 50% PolyEthylene Glycol (PEG)

75 g of PEG was dissolved in 150 ml of dH_2O and was filtered through 0.22 μm filter for sterilization.

5.2.4.2 10x Lithium Acetate (LiAc)

10.2 g of LiAc was dissolved in a total volume of 100 ml in dH_2O to give a 1M solution, and was autoclaved for sterilization.

5.2.4.3 PEG/LiAc

40 ml of 50% PEG and 5 ml of 10x TE buffer, and 5 ml of 1M LiAc were mixed to give a total of 50 ml of PEG/LiAc.

5.2.4.4 1x TE/LiAc

1 ml of 10x TE buffer, 1 ml of 1M LiAc and 8 ml of sterile dH_2O were mixed.

5.2.5.Solutions for X-gal assays

5.2.5.1 Buffer Z

16.1 g of $Na_2HPO_4 \cdot 7H_2O$, 5.5 g of $NaH_2PO_4 \cdot H_2O$, 0.75 g of KCl, 0.246 g of $MgSO_4 \cdot 7H_2O$ were dissolved in 1 L of autoclaved dH_2O, and the solution was adjusted to pH7.0. The solution was filtered for sterilization.

5.2.5.2 X-gal in DMF (stock solution of 20mg/ml)

20 mg of X-gal (5-bromo-5-chloro-3-indolyl-D-galactoside) was dissolved in 1 ml of DMF (N, N-dimethyl formamide) and was stored in an eppendorf tube wrapped in foil at -20°C until use.

5.2.5.3 X-gal Assay Buffer

250 µl of X-gal stock solution and 30 µl of β-mercaptoethanol were mixed in 5 ml of Buffer Z.

5.3. Medium and specific solutions used for *Caenorhabditis elegans*

5.3.1 NGM (liquid and solid medium)

4 g of Sodium Chloride (NaCl), 50 ml of Potassium phosphate (KH_2PO_4) at pH6.0, 3 g of Bactopeptone 21 g of Bacto-agar were dissolved and brought to a total volume of 1 L with dH_2O. In the case of liquid medium, the bacto-agar was omitted. The solution was sterilized by autoclaving. After the sterilization, 1 ml of cholesterol solution in ethanol (5 mg/ml), 3 ml of sterile 1M Magnesium sulphate ($MgSO_4$), 3 ml of sterile 1M Calcium Chloride ($CaCl_2$) and 10 ml of sterile Trace Metal Solution were added. The liquid medium was kept at room temperature. Once the medium was solidified in plates, each plate was seeded with an overnight culture of *E. Coli* strain *OP50* grown in LB. The seeded plates were incubated at 37°C to allow the bacterial lawn to grow on the surface. The plates were stored at 15°C.

5.3.1.1 Cholesterol 5mg/ml in ethanol

500 mg of cholesterol was dissolved in 400 ml of 100% ethanol, and the final volume was brought to 500 ml with 100% ethanol.

5.3.1.2 $MgSO_4$

1M solution of $MgSO_4$ was made in dH_2O and sterilized by autoclaving.

5.3.1.3 $CaCl_2$

1M solution of CaCl2 was made in dH_2O and sterilized by autoclaving.

5.3.2 Aldicarb solution (100mM)

100 mM solution of aldicarb was made in dH_2O and was kept at 4°C till use.

5.3.3 0.5mM Aldicarb plates

5 ml of 100mM aldicarb was added to 1 L of NGM agar and the medium was poured into 6-well plates. The medium was allowed to solidify at room temperature before 15 µl of an overnight culture of *E. Coli OP50* was spotted in the centre of each well. The plates were incubated at 37°C overnight to allow the OP50 to grow. The plates were stored at 15°C.

5.3.4 Bleaching Solution for the egg preparation of *C. elegans*

5 ml of 2N NaOH, 4 ml of 5% alkaline hypochlorite, and 1 ml of dH_2O were mixed together.

5.4. Materials for Molecular Biology

5.4.1 50x TAE (Tris-Acetate-EDTA) buffer

242 g of Tris base, 57.1 ml of acetic acid, and 100 ml of 0.5MEDTA, pH 8.0 was mixed with dH_2O to a total volume of 1L and was sterilized by autoclaving.

5.4.2 1% Agarose gel

1% (weight/volume) of agarose was dissolved in 1x TAE buffer.

5.4.3 6x gel loading buffer for DNA

0.25% (weight/volume) of bromophenol blue, 0.25% (w/v) of xylene cyanol FF, and 30% (w/v) of glycerol in dH_2O was mixed.

5.4.4 Solutions for Mini-preps

5.4.4.1 Resuspension buffer

50mM Glucose, 25mM Tris-HCl (pH8.0) and 10mM EDTA (pH8.0) were mixed to the final concentrations as specified, to a total volume of 50 ml, adjusted with dH_2O.

5.4.4.2 Cell Lysis buffer

1% Sodium Dodecyl-Sulphate (SDS) (pH7.2) and 0.2N NaOH were mixed to the final concentration as specified, to a total volume of 10 ml, adjusted with dH_2O.

5.4.4.3 Neutralization buffer

5.75 ml of Glacial Acetic Acid and 30 ml of 5M Potassium Acetate was mixed to a final volume of 50ml, adjusted with dH_2O.

5.4.5 Solutions for DNA purification by phenol extraction:

5.4.5.1 Chloroform:isoamylalcohol (24:1)

Chloroform was mixed with isoamylalcohol to the ratio of 24:1.

5.4.5.2 Phenol:Chloroform:isoamylalcohol

The mixture of chloroform:isoamylalcohol was added to Phenol to the ratio of 1:1, to make the final solution of Phenol:Chloroform:isoamylalcohol to the ratio of 25:24:1. The mixture was kept in a bottle wrapped with aluminium foil and was stored in the dark at 4°C for minimum 12 hours before use.

5.4.6 10x TE buffer (Tris-EDTA buffer)

10 ml of 1M Tris HCl (pH7.4) and 1 ml of 1M EDTA were mixed to the total volume of 100 ml in dH_2O, to give the final concentration of 100mM of Tris-HCl and 10mM of EDTA. The solution was autoclaved for sterilization.

5.5. Materials for Biochemistry

5.5.1 Running buffer for SDS-PAGE

Final concentrations of 196mM Glycine, 50mM Tris-HCl (pH8.3) and 0.1% SDS (w/v) were mixed in dH_2O.

5.5.2 Transfer Buffer

Final concentrations of 150mM Glycine, 25mM of Tris base and 20% ethanol were mixed in dH_2O.

5.5.3 TBS-Tween

Final concentrations of 150mM NaCl, 10mM Tris-HCl (pH8.0) and 0.2% of Tween-20 were mixed in dH_2O.

5.5.4 3x Laemmli loading buffer for SDS-PAGE

6 mg of bromophenol blue was dissolved in 1 ml of water. 3 ml of 20% of SDS, 2.4 ml of 1M Tris-HCl (pH6.8), 3 ml of 100% glycerol and 1.6 ml of β-mercaptoethanol were added. The total volume was made up to 10 ml with and stored at 4°C.

5.5.5 20% TCA and 5% TCA

20 ml of 100% TCA was mixed with 80 ml of to prepare 20% TCA. 5ml of 100% TCA was mixed with 95 ml of dH_2O to prepare 5% TCA.

6. Methods

6.1 Methods for molecular biology

6.1.1 PCR amplification of the bait inserts

Primers specific for the gene of interest were designed and bought from Sigma or Invitrogen. ESTs specific for the genes of interest (*itsn-1* and *ehs-1*) was kindly provided by Dr. Yuji Kohara from the *C. elegans* consortium. 50 ng of cDNA library of *C. elegans* was used as a template for the amplification of various constructs of rme-1 and that of R10E11.6, whereas a mix of 1 µl of TOPO2.1 clone containing the 5' RACE and 1 µl of TOPO2.1 clone containing the 3'RACE reaction of *reps-1* was used as a template. The amplification of the insert with a polymerase was carried out using PCR machine (maker) in reaction mix in a total volume of 50 µl, set up as follows:

1 µl of 5' oligo (10pmol/µl)

1 µl of 3'oligo (10pmol/µl)

1 µl of template (EST – 100ng/µl)

4 µl dNTPs 2.5mM

5 µl 10x buffer for polymerase

1 µl of polymerase (Taq)

37 µl of sterile water

Final volume of 50 µl

The protocol used for the amplification cycle was as follows:

1 cycle of 5 minutes at 94°C

25 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and1.5minute at 72 °C,

1 cycle of 7 minutes at 72 °C,

and the completed reaction was kept at 4 °C until further use.

6.1.2 Agarose gel electrophoresis

Inserts amplified by PCR reaction were visualised by running 10 µl of the PCR reaction mix in a gel electrophoresis with 1 % agarose gel, loaded alongside a DNA marker *Lambda/Hind III* and *Phi174/HaeIII* or 1kb ladder (Promega). DNA was visualized by Ethedium Bromide (EtBr) under the UV light at 256 nm.

6.1.3 TA cloning

Once the insert of the expected size was amplified by PCR reaction, the insert was first cloned in a cloning vector TOPO2.1, TA cloning kit, Invitrogen. TA cloning reaction was set up as follows:

3 µl of PCR reaction mix

1 µl of Salt solution (provided in the TA Cloning kit, Invitrogen)

1 µl of sterile water

1 µl of TOPO2.1 vector

The total volume of 6 µl was mixed and was incubated for 5 minutes at room temperature.

6.1.4 Transformation of *E. Coli*

The chemical competent *E. Coli* strain *TOP10* (Invitrogen) was transformed with TA cloning reaction as follows. 2 µl of the TA cloning reaction was added to 50 µl of *TOP10* cells and was on ice for 5 minutes. The cells were then heat shocked at 42°C for 30 seconds. 70 µl of SOC medium was added immediately and was incubated at 37°C, shaking at 650 rpm for 1 hour. A total volume of 120 µl of transformed cells was plated on a solid medium LB containing X-gal (80 µl of the stock solution 20mg/ml) and

kanamycin to the final concentration of 50μg/ml assuming that a plate of contains 25 ml of LB.

6.1.5 Extraction of plasmid – Mini-preps

The plasmid was extracted using QIAGEN Mini-prep kit, according to the manufacturer's instruction. When the commercially available kit was not used, the following protocol was used.

TOPO2.1 clones that contain the insert were identified by the fact that the cloning vector is designed such that the insertion of the PCR product into TOPO2.1 vector results in the disruption of the gene encoding β-galactosidase present in the TOPO2.1 vector. Several white transformed colonies grown on the medium containing X-gal were individually inoculated in 2 ml of LB, which contains the appropriate antibiotics, and were incubated at 37°C, constantly shaking at 220 rpm overnight. 1.5 ml of the overnight culture was centrifuged at 14,000 x g for 1 minute. The pellet was resuspended in 250 μl of Solution I, stored at 4°C. 250 μl of the freshly prepared cell lysis buffer, Solution II, was added to the cell suspension and was mixed gently. 250 μl of the neutralization buffer, Solution III, was added and mixed gently. The mixture was incubated on ice for 5 minutes and was centrifuged at 14,000 x g for 10 minutes. The DNA present in the supernatant was extracted using the phenol:chloroform DNA purification protocol, which is described below.

6.1.6 Phenol:chloroform extraction of DNA

An equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was added to the eluate, vortexed and centrifuged. The upper later corresponding to the fraction containing the DNA was mixed with chloroform:isoamylalcohol (24:1). The mixture

was vortexed and centrifuged. The DNA contained in the upper fraction was then precipitated with 5M sodium acetate and 100% ethanol.

6.1.7 Precipitation of DNA with Sodium Acetate/Ethanol

A 1/10 volume of 5M Sodium Acetate and a twice the volume of 100% ethanol was added to the fraction containing DNA. The mixture was vortexed briefly and was left to freeze in a dry ice bath for 15 minutes. The DNA was precipitated by centrifugation at 14,000 x g for 15 minutes at 4°C. The pellet was washed with an equal volume of 70% ethanol, centrifuged 14,000 x g for 10 minutes at 4°C. The pellet was left to dry for 5 to 10 minutes and was resuspended in resuspended in 50 µl of TrisHCl 10mM pH 8.0, containing 0.1 mg/ml RNase A (Promega).

6.1.8 Diagnostic digestion of the plasmid extracted

200-300 ng of the plasmid and 2units of the appropriate restriction enzymes in the suitable buffer was mixed and added up to a total volume of 20 µl with dH_2O. The digestion mix was incubated at 37°C for 90 minutes. 4 µl of 6x loading buffer was added to the total volume of 20 µl and was loaded onto 1% agarose gel as described in the previous section 6.1.2 on the agarose gel electrophoresis.

6.1.9 Preparation of the cloning vector and the insert

The TOPO2.1 clone that was found to be positive for the insert was digested with the specific restriction enzymes used as the cloning sites to release the insert. At the same time, the cloning vector was digested with the same restriction enzymes to prepare the cloning site. 3 µg of the plasmid with insert, and 5 µg of the cloning vector was each digested with 3 units of the appropriate restriction enzymes in the suitable buffer at the optimum temperature, which was commonly 37°C, for a total of 1 hour. Usually 2 units

of each enzyme were used in the first 30 minutes and the other unit was added in the second 30 minutes. The total volume of digestion was made up to 80 µl with dH_2O. After 1 hour of digestion, 2% of the total volume of the digestion mix was loaded in 1% agarose gel electrophoresis to ensure that the digestion was efficient.

6.1.10 Calf Intestinal Phosphatase (CIP) treatment of the cloning vector

The overhanging nucleotides at the restriction sites were dephosphorylated to prevent the re-closure. The plasmid in the 80µl of the digestion mix was purified from the enzymes and the buffer by phenol:chloroform extraction and precipitated with sodium acetate/ethanol as described in the sections 6.1.6 and 6.1.7, respectively. To 20 µl of the plasmid extracted, 1 unit of CIP (New England BioLabs) was added with its appropriate buffer. The total volume was made to 50 µl with dH_2O and was incubated at 37°C for 1 hour. The plasmid was purified from CIP and its buffer by phenol:chloroform extraction, followed by sodium acetate/ethanol precipitation. The precipitate was resuspended in 15 µl of sterile dH_2O and was stored at -20°C until used for ligation.

6.1.11 Gel extraction of DNA

All of the remaining digestion mix (16 µl) remaining from the step described in Section 6.1.8, was then run in 1% agarose gel electrophoresis to isolate the insert. The band corresponding to the expected size of the insert was excised. The band was visualized with EtBr under the UV light at 70% emission, with the gel piece kept on the plastic tray for running the gel, in order to minimize the damage by the UV. The DNA in the gel band was extracted from the agarose gel piece by centrifugation through a packed glass wool. The DNA was further purified by phenol:chloroform followed by

precipitation with sodium acetate/ethanol, as described in the sections 6.1.6 and 6.1.7, respectively. The precipitate was resuspended in 10 µl of sterile dH_2O instead of 20 µl.

6.1.12 Ligation

1 µl of insert and 1 µl of the cloning vector prepared were loaded in 1% agarose gel to quantify approximately. The cloning vector and the insert was mixed to the ratio of 1:4, in excess of the insert, and was kept on ice whilst the ligation buffer and 1 unit of Fast Ligase (Promega) was added and made to a total volume of 10 µl with dH_2O. At the same time, a negative control was prepared by incubating the vector without the insert with the ligase. The mixture was shaken briefly and was incubated at room temperature for 5 minutes. 50 µl aliquot of chemical competent *E. Coli* cells *Select 96* (Promega) was immediately transformed with 3 µl of each ligation mix by heat shock as described in Section 6.1.4. The number of colonies transformed by the ligation of the vector plus insert and that of vector only were compared. If the ratio of vector plus insert: vector only was more than 4:1, minimum of 4 colonies were inoculated in 2 ml of LB for mini-preps as described in section 6.1.5.

6.1.13 Large-scale preparation of plasmid

The clone that was confirmed to contain the plasmid of interest by diagnostic digestion of mini-prep was inoculated in 500 ml of LB medium with the appropriate antibiotic, and was incubated overnight at 37°C, shaking constantly at 220 rpm/min. 500 ml of the overnight culture was centrifuged at 3,000 rpm for 10 minutes. The plasmid was extracted using QIAGEN Maxi-prep kit, according to the manufacturer's instructions.

6.2. Methods for Y2H Screening

6.2.1 Preparation of Competent Yeast cells

20 ml of YPD medium was inoculated with a single colony of *MaV203* yeast cells and was incubated at 30°C, constantly shaking at 220 rpm for 18 hours. 480 ml of YPD was added and the culture was incubated at 30°C, constantly shaking at 220 rpm for 3-4 hours. When the Optical Density at 600 nm (OD_{600}) of the culture reached within the range of 0.5 ± 0.1, the culture was centrifuged at 3,0000 rpm for 5minutes. The pellet was resuspended in 50 ml sterile dH_2O and was centrifuged at 3,000 rpm for 5 minutes. The pellet was resuspended in 10 ml sterile dH_2O. The competent cells were aliquoted into 50 µl per transformation.

6.2.2 Small-scale Co-Transformation of Competent Yeast cells MaV203

100 ng of each plasmid (pPC86 and pDBLeu constructs) were added to 50 µl of competent yeast cells and were kept on ice. 1 ml of salmon sperm (Invitrogen) was added as a carrier DNA. 300 µl of PEG/LiAc and was mixed well. The cells suspended in PEG/LiAc were immediately placed a water bath at 30°C for 20 minutes, where the tube was shaken to mix every 10 minutes. The aliquot was transferred to a water bath kept at 42°C for 15 minutes. The yeast cells were collected by centrifugation at 7,000 x g for 30 seconds. The pellet of cells were resuspended in 100 µl of sterile dH_2O, were plated on a selective SC medium lacking Tryptophan and Leucine, and were incubated at 30°C for 48 hours.

6.2.3 Preparation of the masterplates for testing the self-activation of *LacZ* gene expression by the bait

Five of the co-transformants were streaked on to a plate SC –L/-W on a grid as shown in the schematic diagram below alongside the control strains A, B, C, D and E provided in the Yeast Two Hybrid (Invitrogen).

A Schematic diagram to show the grid on which the co-ransformants were streaked on a plate containing the selective medium alongside the control strains:

Control strains	pDBLeu -bait/ pPC86
A	1
B	2
C	3
D	4
E	5

The control strain A is a negative control that is a co-transformant of an empty bait vector and an empty prey vector. Each of the control strain B, C, D, and E express interactors with the increasing strength of interaction. The control strains are described in Table 1. The masterplate was incubated at 30°C for 48 hours.

Table 1 Description of the Yeast Control strains A-E used in the Y2H Screenings
(Taken from the protocol for ProQuestTM Two-Hybrid System, Invitrogen).

Control Strain	Resident Plasmids	cDNA insert	Interaction strength	Reference
A	pPC97 pPC86	no insert	none	Chevray, P. and Nathans, D. (1992) *Proc. Natl. Acad. Sci. USA* 89: 5789
B	pPC97-RB	human RB Acc. No. M28419 amino acids 302-928	weak	Vidal, M. (1997) The Reverse Two-Hybrid System in the *Two-Hybrid System* , (Bartel, P. and Fields, S., eds.),Oxford University Press, New York, 109 Vidal, M., et.al., (1996) *Proc.Natl.Acad.Sci.USA* , 93:4091
	pPC86-E2F1	human E2F1 Acc. No. M96577 amino acids 342-437		
C	pPC97-CYH2^{*dDP}	Drosophila DP Acc. No. X79708 amino acids 1-377	moderately strong	Du, W., Vidal, M., Xie, J-A., Dyson, N. (1996) *Genes and Dev* . 10:1206
	pPC86-dE2F	Drosophila E2F Acc. No. U10184 amino acids 225-433		
D	pPC97-Fos	rat cFos Acc. No. X06769 amino acids 132-211	strong	Chevray, P. and Nathans, D. (1992) *Proc. Natl. Acad. Sci. USA* 89: 5789
	pPC86-Jun	mouse cJun Acc. No. X12761 amino acids 250-325		
E	pPC97-Fos	rat cFos Acc. No. X06769 amino acids 132-211	very strong	Fields, S., and Song, O-K. (1989) *Nature* 340:245
	pPC86-Jun	mouse cJun Acc. No. X12761 amino acids 250-325		

6.2.4 Self-activation Test for bait constructs by X-gal Assay

The masterplate prepared as described in Section 6.2.3 was replica plated onto a nitrocellulose membrane placed on a plate of YPAD medium, and was incubated at 30°C for 16 hours. The yeast cells grown on the nitrocellulose membrane were frozen at -80 °C for 20 minutes. The nitrocellulose membrane was then transferred onto a layer of two filter papers soaked in 5 ml X-gal assay buffer. Any excess buffer was removed and the membrane was incubated at 37°C for 2 hours. The expression of *LacZ*, which is detectable by the appearance of blue colonies due to the conversion of X-gal, was checked after 2 hours, and was left incubated at 37°C for 24 hours.

6.2.5 TCA Precipitation of proteins from yeast liquid culture

The five co-transformants streaked onto the masterplate were inoculated in 5 ml of SC – L/-W and were incubated at 30°C shaking for 24 hours. The cells were harvested by centrifugation at 3,000 x g for 5 minutes. The pellets were resuspended in 1.5 ml 20% TCA, were transferred to 2 ml-eppendorf tubes and were centrifuged at 14,000 x g for 1 minute. The pellet was resuspended in 200 µl 20% TCA. 30 µl volume of acid-washed glass beads (425-600 µm) was added to each tube and was vortexed for 3 minutes. The glass beads were allowed to settle at the bottom of the tube and the supernatant was transferred to a clean eppendorf tube. 400µl of 5%TCA was added to each tube and were centrifuged at 3,000 x g for 10 minutes. The pellet was resuspended in 25 µl of Laemli buffer and 12.5 µl of TRIS was added to adjust the pH suitable for polyacrylamide gel electrophoresis. The protein extracts were denatured at 99°C for 5 minutes and were centrifuged at 3,000 x g for 10 minutes. 20 µl of the supernatant was loaded in 10% polyacrylamide gel.

6.2.6 SDS-PAGE

Polyacrylamide gel was prepared from a 40 % mix of acrylamide : bisacrylamide to the ratio of 30:1. 10% Ammonium persulphate (APS) and tetramethylethylenediamine (TEMED) were used as polymerizing catalysts. The proportions of solutions to prepare 8% - 15% polyacrylamide gels are shown in the table below:

Separating gel mix (ml):	**8%**	**10%**	**15%**
acrylamide mix	6	7.4	11.25
1.5M Tris pH 8.8	7.4	7.4	7.4
dH_2O	15.9	15.4	10.65
10% SDS	0.3	0.3	0.3
10% APS	0.3	0.3	0.3
TEMED	0.03	0.03	0.03
TOTAL volume	30	30	30

Stacking gel mix (ml):

Acrylamide mix	1.7
1M Tris pH6.8	1.25
dH_2O	6.8
10% SDS	0.1
10% APS	0.1
TEMED	0.01
TOTAL volume	10

20 µl of TCA protein precipitation of each co-transformants were loaded in the polyacrylamide gel and the electrophoresis was run at 100 V for 90 minutes to 2 hours, until the loading dye ran out of the gel.

6.2.7 Western Blot

The proteins in the polyacrylamide gel were then transferred onto a nitrocellulose membrane that was sandwiched by layers of filter paper and a transfer sponge (as shown in the schematic diagram below).

A schematic diagram to show the set-up of the layers of a nitrocellulose membrane and a polyacrylamide gel for the transfer of proteins:

Negative electrode	-
Transfer sponge	XXXXXXXXXXXXXXX
Filter paper	----------
Polyacrylamide gel	oooooooooooooooooooo
Nitrocellulose membrane	**********
Filter paper	----------
Transfer sponge	XXXXXXXXXXXXXXXX
Positive Electrode	+

The transfer was carried out either at 30 V overnight at room temperature, or at 100 V at 4°C with ice block for 1 hour.

The proteins the nitrocellulose membrane were visualized by Ponceau staining to ensure that the transfer was efficient.

The unspecific binding was blocked with 5% milk in buffer TBS-Tween 0.2% for 30 minutes at room temperature. The primary antibody, anti-GAL4DBD antibody diluted in 5% milk/TBS-Tween to the ratio of 1:1,000 was added to the membrane and left incubated rocking at room temperature for 1 hour. The unbound primary antibody was washed off with TBS-Tween 0.2% with four changes of buffer for a total of 30 minutes. The secondary antibody, anti-mouse antibody diluted in TBS-Tween 0.2% to the ratio of 1: 20,000 was added to the membrane and left incubated rocking for 30 minutes. The unbound secondary antibody was washed off with TBS-Tween 0.2% with four changes of buffer for a total of 30 minutes. The ECL solutions (Amersham-Biopharmaceuticals) were used for the detection of anti-GAL4 DBD signals.

6.2.8 3AT Assay

3AT assay was an assay to determine the appropriate 3AT concentration required for titrating the basal HIS3 expression of the co-transformants. The masterplate, prepared as described in Section 9.3, was replica plated onto a series of SC –L/-W/-H plates containing varying concentration of 3AT from 0mM to 100mM. The plates were incubated at 30°C for 24 hours. The plates were replica cleaned until no residue of cells were visible, and were incubated at 30°C for 48 hours. The lowest concentration of 3AT at which the growth of the co-transformants was comparable to control strain A, the negative control, was determined as the concentration required for the Y2H screening with the bait.

6.2.9 Preparation of the cDNA library

The glycerol stock containing the cDNA library clones (purchased from Invitrogen) was inoculated in 500 ml of LB added with 50μg/ml of Ampicillin. The culture was incubated at 30°C for 16 hours. After the incubation for 16 hours, the culture was centrifuged and the pellet was resuspended in 30 ml of cell resuspension buffer P1

(QIAGEN). The cDNA was purified using a protocol for the large-scale purification of DNA, according to the manufacturer's instruction by QIAGEN. The purified cDNA library was divided into aliquots of 10 μg and the aliquots were kept at -20°C till use.

6.2.10 Co-transformation of library scale competent yeast cells MaV203 with a bait and a cDNA library

The 500 μl aliquot of *Mav203* cells (purchased from Invitrogen) that was stored at -80°C was placed in a water bath set at 30°C, gently shaking for 90 seconds. 250 μl was transferred to a 50 ml-falcon tube and was kept at room temperature. 10 μg of pDBLeu-bait and 10 μg of pPC86-cDNA library were added to 250 μl of competent yeast cells and were mixed well. 1.5 ml of PEG/LiAc was added to the cells and the mixture was swirled gently until the cells were suspended in PEG/LiAc homogenously. The suspension of yeast cells was incubated in a water bath at 30°C for 30 minutes, shaking every 10 minutes. The suspension of yeast cells was then heat shocked at 42°C for 20 minutes, shaking after 10 minutes. The cells were collected by centrifugation at 4,000 x g for 5 minutes and were resuspended in 1.5 ml of 0.9% sodium chloride solution. 5 μl of the cell suspension was added to each of 95 μl of water (the dilution factor 1: 800) and to 995 μl of water (the dilution factor 1: 8,000). The cells were resuspended well, and 100μl of each resuspensions were plated on SC –L/-W. These plates were used to calculate the efficiency of co-transformation. 150 μl of the suspension was plated per 16 cm-diameter plates of SC –L/-W/-H/+3AT. The plates were incubated at 30°C for 72 hours to 96 hours. The co-transformation with each bait used in the Y2H screening was repeated until the total co-transformation efficiency reached 1 x 10^6 colonies to ensure that the complexity of the whole genome represented by the cDNA library was covered.

6.2.11 Preparation of the masterplates for Y2H screening

The yeast co-transformants that grew on the screening plates were checked and marked after 48 hours, 72 hours and 96 hours. The clones that grew in the selective medium for the screening were streaked onto masterplates in grid alongside the control strains as shown in the schematic diagram below. The control strains are described in detail in Table 1 Description of the Yeast Control strains A-E used in the Y2H screenings.

Legends:

C-: clone used as the negative control, aco-transformant of the bait and the empty prey vector (pDBLeu-ITSN-1/pPC86 or pDBLeu-EHS1 123/ pPC86)

C-1, C-2, C-3: Three clones used as the negative controls, which were three co-transformants of the bait and the empty prey vector (pDBLeu-RME-1/pPC86, pDBLeu-Reps1/pPC86, or pDBLeu-R10E11.6/pPC86)

The clones, which were co-transformed with the bait constructs and the empty prey vector, were used as the specific negative control for the clones from the screenings. In comparison, the control strain A, which is a clone transformed with plasmids pPC97 and pPC86, both with no insert (refer to Table 1 Description of the Yeast Control strains A-E used in the Y2H screenings in Section 6.2.3) was used as the general negative control for the X-gal assay. The other control strains B, C, D, and E, which are also described in detail in Table 1 in Section 6.2.3, were used as references to indicate the different strengths of interactions observed in the X-gal assays of the clones from the screenings.

The schematic diagram to show the grids for the masterplates used in the Y2H screenings:

Masterplates for ITSN-1 and EHS-1 123 Y2H screenings

A	C-	1	2	3	4
B	5	6	7	8	9
C	10	11	12	13	14
D	15	16	17	18	19
E	20	21	22	23	24

Masterplates for RME-1, Reps1, R10E11.6 Y2H screenings

A	1	2	3	4	5	
B	6	7	8	9	10	C-1
C	11	12	13	14	15	C-2
D	16	17	18	19	20	C-3
E	21	22	23	24	25	

6.2.12 X-gal assay to test the *LacZ* expression of positive clones

The clones grown on the masterplates were replica plated onto nitrocellulose membrane and tested for *LacZ* expression by X-gal assay as described in Section 6.2.4.

6.2.13 Test for *URA3* expression

The masterplates were replica plated onto plates lacking Uracil (SC -L/-W/-U) to test for the expression of *URA3*. The plates were incubated at 30°C for 24 hours and were replica cleaned until there were no residues of yeast cells visible. The plates were incubated at 30°C for 48 hours before the growth of each clone was observed.

6.2.14 Extraction of plasmid from yeast positive clones

The clones, which were shown to be positive for the expression of the reporter genes, were each inoculated in 5 mL of SC –L/-W, and were incubated shaking at 30°C for 30 hours. The cells were harvested by centrifugation at 4,000 x g for 5 minutes at room temperature. The cell pellet was resuspended in 250 µL of resuspension buffer P1 (QIAGEN). 30 µL of acid-washed glass beads were added and the cell suspension was vortexed for 5 minutes. The glass beads were allowed to settle and the supernatant was transferred to another eppendorf tube. 250 µl of cell lysis buffer P2 (QIAGEN) was

added, mixed gently and incubated at room temperature for 5 minutes. 350 µl of the neutralization buffer P3 (QIAGEN) was added to the cell lysate, mixed gently and centrifuged immediately at 14,000 x g for 10 minutes. The supernatant was loaded onto QIAGEN mini-prep column and was centrifuged through the column for DNA to bind to the column, at 14,000 x g for 1 minute. The column was washed with 750 µl of the wash buffer provided, by centrifuging through at 14,000 x g for 1 minute. The wash buffer was removed, and the excess wash buffer left on the column was removed by centrifugation at 14,000 x g for 2 minutes. The yeast plasmid bound on the column was eluted with 15 µl of elution buffer EB.

6.2.15 Transformation of *E. Coli* with plasmid extracted from yeast.

The plasmids extracted from yeast cells were passed to *E. Coli* by transformation by heat shock in order to obtain a plasmid preparation suitable for digestion by restriction endonucleases. 5 µl of the yeast mini-prep prepared in Section 6.2.14 was added to 50 µl of *E. Coli Select 96* competent cells (Promega). The competent *E. Coli* was transformed as described in Section 6.1.4.

6.2.16 Identification of the cDNA insert

The digestion was loaded in 1% agarose gel electrophoresis and visualised by Ethedium Bromide staining. The *E. Coli* clones with an insert were sequenced for the identification of the cDNA insert. The plasmid purified as described in Section 6.1.5 was sequenced using an oligo specific to the upstream of the multiple cloning site in the prey vector pPC86 of the sequence as follows:

5' TATAACGCGTTTGGAATCACT 3'

Sequencing was carried out using only the 5' oligo as partial sequence was sufficient for its identification from the non-redundant database. The partial DNA sequence of the cDNA was then translated using the Expasy Translate Tool that is available on the Internet at :http://www.expasy.ch/tools/dna.html.

In order to ensure that the cDNA fragment was cloned in-frame with GAL4AD, the DNA sequence was translated into amino acid sequence, starting from the DNA sequence that encoded the last part of the GAL4AD, as highlighted in an example of the following sequence:

```
>6428.7-1-ppc86f.07.abi  885   0  885 ABI
AGGNNATGTTTANTACCACTACAATGGATGATGTATATAACTATCTATTCGATGATGAAGATACCCCAC
CAAACCCAAAAAAAAGAGGGTGGGTCGACCCACGCGTCCGGTTTGAGGCGGGGAGCAAACATGTCGATTT
CGACGATTCGCCGGCAAGTGAAGAACGTCGCCTATAATTTCTCGGATGCACAAGTAAAGGTTCGCGAAG
CCACAAGCAACGATCCATGGGGACCATCCACAGCGCTCATGTCCGAAATTGCCGACTTGACGCACAATC
CAATGGCGTTCACTGAGATTATGTCAATTGTTTGGAAGCGATTAAATGATTCTGGCAAAAACTGGCGAC
ACGTGTACAAAAGTCTGGTGCTTCTCGATTTTCTCATCAAATGCGGCCACGAGAAGGTCGCGCAACAAT
GCAGAGAGAACGTGTTCACCATTGAAACACTCAAGGATTTCCAGCATGTGGAAGACAATCGGGATCAGG
GCTTGAACATTCGAGAGAAAGCAAAGCAGATCACTTCACTGCTCTCCGACGATGAACGCTTGAAAAACG
AAAGAACACGCTTCATCCTCACAAGAAACAAATTCAAGCAAAACAATCCAGGACCGGTTGGAGCGGAAA
GTCGTCGCAGCAATCGTCATCATGTCACCGACGCGTCTCTTGACCCGAGTACGAAGATGCTCGTCCATC
TACCGCCACGAGGAAGAGATGCAGCTTCAAATTGCTCTTGCTCTTTCACGAGANGAATGCGAAAAGGNC
GACNANATGCGCAAGAGCGATGATGCNCGCCTTCAAATGGCNCNGGAAGANTCCCAAAAGCTTCAGGAT
GCCGNTCCCTCCCAACNACAANCANGGTACGTNAGCANCGGANATTAACCNATCCCC
```

An example of the translation of the sequence above is shown below:

```
5'3' Frame 1

DDVYNYLFDDEDTPPNPKKEGGSTHASGLRRGANMSISTIRRQVKNVAYNFSDAQVKVREATSNDPWGPST
ALMSEIADLTHNPMAFTEIMSIVWKRLNDSGKNWRHVYKSLVLLDFLIKCGHEKVAQQCRENVFTIETLKD
FQHVEDNRDQGLNIREKAKQITSLLSDDERLKNERTRFILTRNKFKQNNPGPVGAESRRSNRHHVTDASLD
PSTKMLVHLPPRGRDAASNCSCSFTRXMRKXRXXAQER-
CXPSNGXGRXPKASGCXSLPXTXXVRXXRXLTXP
```

The amino acid sequence till the end of the reading frame (-), shown highlighted in the sequence above was taken and was searched against the *C. elegans* non redundant database using BLASTP (Protein BLAST).

6.2.17 Re-transformation assay

The cDNA inserts, which were identified as a putative interactor of the bait, were re-transformed with the bait into 50 µl of *Mav203* competent cells as described in Section 6.2.2. The yeast colonies were streaked onto masterplates of SC –L/-W, alongside the control strains as shown in the schematic diagram below, and the plates were incubated at 30°C for 48 hours. The control strains that were used are described in detail in Table 1. The expression of the reporter gene *LacZ* by the re-transformants were tested using the X-gal assay, as described in Section 6.2.4.

A Schematic diagram to show the grid used to streak the yeast colonies on a plate containing the selective medium alongside the control strains:

Masterplates for re-transformation assays

Control strains	Bait/ cDNA 1	Bait/ cDNA 2	Bait/ cDNA 3	Bait/ cDNA 4	C-
A	1	1	1	1	
B	2	2	2	2	C-1
C	3	3	3	3	C-2
D	4	4	4	4	C-3
E	5	5	5	5	

For the description and the explanation of the control strains A-E and of the clones used as the negative controls (C-1, C-2 and C-3) in this assay, refer to Table 1 Description of the Yeast Control strains A-E used I the Y2H Screenings in Section 6.2.3 and to Section 6.2.11.

6.2.18 Rapid Amplification of cDNA ends (RACE) for Y39A6A.38 (5'RACE and 3' RACE)

5' RACE and 3' RACE were carried out using the FirstChoice™ RLM-RACE Kit (Ambion Inc, Applied Biosystems). The reactions were set up according to the manufacture's instructions, using the genomic DNA of *C.elegans* as template. The PCR

products were cloned into TOPO2.1 vector using the TA cloning kit, as described in the section 5.1.3. 50 ml of chemical competent *TOP10* cells purchased from Invitrogen was transformed with 3ml of the inserts ligated into the TOPO2.1 vector, as described in the section 5.1.5. The plasmid was extracted from the transformed colonies by mini-prep as described in the section 5.1.5. The inserts in the extracted plasmids were sequenced using primers specific for TOPO2.1 vectors.

6.2.19 Quantitative PCR (qPCR) amplification of cDNA inserts from the cDNA library

(The primers were designed by Dr. Loris Bernard of the Real Time PCR Unit, Cogentech at IFOM, Milan, Italy. The qPCR was carried out by Cogentech. The selection of the genes encoding NPF-containing proteins were assisted by Dr. Stefano Confalonieri, IFOM, Milan, Italy).

16 genes encoding NPF-containing proteins from the *C. elegans* genome were selected in order to test their level of representation in the cDNA library used in the Y2H screenings.

Briefly, the cDNAs of these selected genes were amplified from the cDNA library using specific primers and the level of fluorescence emitted by the incorporation of SYBR-Green® in the amplicons were quantified. Prior to the qPCR analysis by the facility, the concentration of the magnesium chloride in the PCR reaction buffer was optimized in the range 0mM to 1.5mM for each set of primers in order to have specific amplifications. 3 genes (indicated by arrows in the Table 2) that were initially chosen were discarded because many unspecific bands were observed despite the various conditions attempted.

Table 2 Sixteen genes selected for the qPCR analysis to test the level of representation in the cDNA library used in the Y2H screenings.

	WormBase Acc	Gene Name	RefSeq	Bait	freq	Other Bait ?	Freq	Tot Freq	Validation	Bind motif?	Description
Fished at HI Freq.	T04C10.2	EPN-1	NM_078057	EHS-1	20	INT-1	18	38	re-trasf.	4 NPF	liquid facets (51.0 kD) (XP436)
	C09H6.2	LIN-10	NM_059825	EHS-1	11	INT-1	4	15	re-trasf.	2 NPF	abnormal cell LINeage LIN-10, amyloid beta precursor protein-binding family A-like, controls cell polarity (102.3 kD) (lin-10)
	Y63D3A.5	TFG-1	NM_061061	EHS-1	13	INT-1	2	15	re-trasf.	1NPF	human TFG related (49.7 kD) (tfg-1
	F29C12.1	PQN-32	NM_064384	EHS-1	4	INT-1	5	9	re-trasf.	2 NPF	Prion-like Q/N-rich domain protein (66.0 kD) (pqn-32)
Fished at LOW Freq.	F41G4.2	CAS-1	NM_078313	EHS-1	1			1	re-trasf.	1NPF	adenylyl Cyclase ASsociated protein Homolog (53.0 kD) (cas-1)
	R0E12.1	ALX-1	NM_066812	EHS-1	1	RME-1	1	2	re-trasf.	1 NPF	prion-like Q/N-rich domain protein PQN-58, Prion-like Q/N-rich domain protein, vertebrate ALG-2 / programmed cell death 6 interacting protein homolog YNK1
	T05E7.5	vet-1	NM_059386			INT-1	4	4	re-trasf.	1 NPF	very Early Transcript VET-1, anti-apoptotic factor 1 like (vet-1)
	F28H1.2	cpn-3	NM_058881	EHS-1	2			2	re-trasf.	1 NPF	CalPoNin (cpn-3)
NEG NPF >1	M01D7.2	scm-1	NM_058510		0		0	0	None	2 NPF	Caenorhabditis elegans secretory Carrier associated Membrane protein
	R08C7.9	4F204	NM_068164		0		0	0	None	4 NPF	cyclin-like F-box family member (4F204)
	R06F6.2	2L78	NM_063921		0		0	0	None	4 NPF	vacuolar protein sorting 11 (108.4 kD) (2L78) [Caenorhabditis elegans]
	C32E8.10	UNC-11	C32E8.gc10		0		0	0	None	5 NPF	AP180-like adaptor protein family member, UNCoordinated locomotion UNC-11 (60.2 kD) (unc-11)
NEG NPF =1	F18C12.2	rme-8	NM_059821		0		0	0	None	1 NPF	rme-8 j-domain containing protein, required for endocytosis, Receptor Mediated Endocytosis RME-8 (259.2 kD) (rme-8)
	R13A5.11	3l680	NM_066265		0		0	0	None	1 NPF	protein phosphatase family member (3l680)
	JC8.10	unc-26	NM_171423		0		0	0	None	1 NPF	synaptojanin, UNCoordinated locomotion UNC-26, (123.4 kD) (unc-26)
	B0285.1	3F429	NM_065472		0		0	0	None	1 NPF	protein kinase (3F429) Putative cell division protein kinase 9 homolog

6.3 Methods for *In Vitro* Pulldown assays

(This part of the project was carried out by Dr.Francesca Senic-Matuglia, IFOM, Milan, Italy)

6.3.1 Expression of FLAG-tagged EH proteins

Full length C. elegans' EH proteins were cloned in-frame with a FLAG-tag in pCDNA vectors. For genes such as *ehs-1* and *rme-1*, where several isoforms are reported, the sequences of the isoform a of both genes were used, whereas *itsn-1* and *reps-1* have only one reported isoform each. The FLAG-tagged EH proteins in pCDNA vectors were expressed in mammalian HEK293T Phoenix cell line, by transfection with Calcium phosphate. The over-expression in mammalian cell line was chosen in order to obtain a large quantity of the FLAG-tagged EH proteins.36 hours after transfection, cells were lysed in JS buffer (Hepes 50 mM pH 7.4, NaCl 150 mM, Glycerol 10%, Triton X100 1%, $MgCl_2$ 1,5 mM, EGTA 5 mM, and Protease Inhibitor cocktail set III EDTA free from Calbiochem) and clear lysates were obtained by centrifugation at 14,000 rpm. Expressions of the FLAG-tagged proteins were verified by Western blot using anti-FLAG M2 antibody (Sigma).

6.3.2 Preparation of the GST-fusion constructs

The cDNA inserts that were isolated from the clones showing an interaction with EH proteins in the Y2H screenings were cloned in pGEX-6-P2 vector, in order to express them as GST-fusion proteins. If different lengths of DNA inserts for one putative interactor had been identified, the shortest insert was chosen. *E. Coli* strain *BL21* was then transformed with the pGEX constructs.

A single colony of transformed *E. Coli BL21* was inoculated in 50 ml of LB with Ampicillin and was incubated overnight at 37°C, shaking at 220 rpm. 20 ml of the overnight culture was added to 1 L of LB-Amp. Diluted culture was incubated at 37°C,

shaking at 220 rpm, until the absorbance at 600nm (A_{600}) reached 0.6. IPTG was then added to the culture to the final concentration of 1mM to induce the expression of GST-fusion proteins. The culture was left to express for 5 hours at 30 °C. Bacterial cells were collected by centrifugation at 5,500 x g for 15 minutes at 4 °C. The supernatant was discarded and the pellet was washed with 50 ml cold PBS. After another centrifugation step, the bacteria pellet was resuspended in 10 ml of PBST (PBS, 0.1% of tween20, and the protease inhibitors cocktail purchased from Calbiochem). The cells were disrupted by sonication on ice (30 seconds at 30% of intensity for 3 cycles). Lysate was clarified by 30 min centrifugation at 20,000 x g at 4 °C. GST proteins were then purified by incubation with 1 ml slurry of Glutathione Sepharose 4B beads (75% slurry - Amershampharmacia Biotech) on wheel for 2 hours at 4 °C. The beads were then washed 5 times with cold PBST and twice with conservation buffer (HEPES 20 mM pH 7.4, NaCl 100 mM, glycerol 10%). The beads carrying the GST purified proteins were then divided into aliquots and were stored at – 80 °C.

6.3.3 Preparation of the Glutathione Sepharose beads

The matrix of sepharose beads was centrifuged at 500 x g for 5 minutes, and the supernatant was carefully removed. The beads were washed by adding 10ml of cold 1 x PBS and inverting gently the tube five times. The mixture was centrifuged at 500 x g for 5 minutes and the supernatant was removed. The washing steps as above were repeated three times.

6.3.4 *In vitro* pulldown assays

In vitro binding assay were performed incubating 120 pmol of GST proteins (previously normalised on a SDS-PAGE stained with Coomassie staining) with the lysate harbouring the *C. elegans* EH proteins for 2 hours at 4 °C. The quantity of the lysates used in the pulldown assays were normalized on the amount of FLAG-tagged EH

proteins detected in each lysate by the Western blotting with anti-FLAG antibody. GST-NPY, which is a GST fused with a peptide sequence isolated from human Epsin, which contains the mutation of the NPF motif into NPY, and GST alone were used as negative controls. Beads were then rapidly washed three times with 1 ml JS buffer and 50 µl of loading buffer was added. After separation on SDS-PAGE and transfer onto nitrocellulose, the presence of EH proteins in the pulldowns were analysed by Western blot using the anti-FLAG antibody and ECL (Amersham-GE Healthcare) as Western blotting detection reagents.

6.4. Methods for *Caenorhabditis elegans*

6.4.1 Preparation of cultured worms prior to the egg preparation

A single colony of *OP50* from a plate on which the glycerol stock of *OP50* was streaked on 18 hours prior, was inoculated in 20 ml of LB, and was incubated shaking at 37°C overnight. 100 µl of the overnight culture of *OP50* was plated on 15 plates of NGM agar medium. The plates were incubated overnight at 37°C. The plates were left to cool at room temperature for 1 hour before they were transferred at 15°C for 2 hours. The plates were divided into five sets of 3 plates. From a plate of mixed-stage population of *N2* strain, 6 adult worms were placed on one plate (Plate 1), 6 worms of L3/L4 staged worms on the second plate (Plate 2), and 6 worms of L1/L2 worms were placed on the third plate (Plate 3). The procedure was repeated for the other four strains of *C. elegans*, namely; *itsn-1(ok268)*, ehs-1*(ok146)*, *rme-1(b1045)* and *reps1(tm2156)*. Plates 1, Plates 2 and plates 3 of each strain were incubated at 15°C for 5 days, 6 days and 7 days, respectively.

6.4.2 Egg preparation of *C. elegans*

For each plate of five strains of *C. elegans* prepared, the adult worms with eggs were collected in 5 ml of water. 9 ml of water was added and worms were collected by centrifuged at 1,500 x g for 2 minutes. The pellet of worms was resuspended in 4 ml of water. 4 ml of bleaching solution was added to the resuspension of worms and the mixture was shaken vigorously for 5-6 minutes. When the bodies of adult *C. elegans* were no longer visible in the resuspension, 6ml of water was added immediately and centrifuged at 1,500 x g for 2 minutes. The pellet of eggs was washed with 14ml of water three times. The pellet was resuspended in 1 ml of sterile water and was incubated at 15°C for 15 hours in order to synchronize the population.

6.4.3 RNA *interference* of putative interactors by feeding

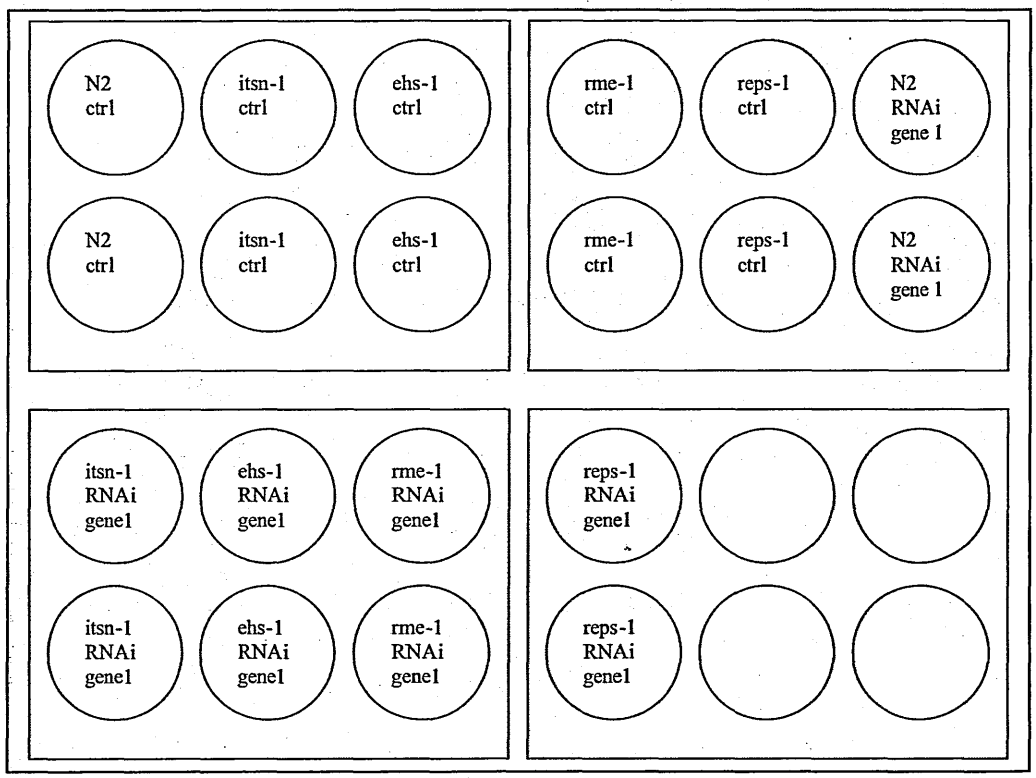
The bacterial strain *E. Coli HT115* transformed with the plasmid L4440 containing cDNA that encodes for a putative interactor was inoculated in 10 ml of LB+Ampicilin, taken from the RNA*i* library (Geneservice Ltd), and incubated shaking overnight at 37°C.

The overnight culture was induced with final concentration of 1mM IPTG and of 2μg/ml of tetracycline and was left incubating at 37°C, shaking for 4 hours. The induced culture was harvested at 4,000 x g for 7 minutes and resuspended in 500 μl of LB+Ampicilin. 100 μl of the resuspension was plated per feeding plate and was incubated overnight at 37°C. 150 μl of a resuspension of *C. elegans* synchronized at L1 stage from the egg preparation was added to the feeding plate and was incubated at 15°C for 4-5 days until the *C. elegans* reached young adulthood.

6.4.4 Aldicarb Assay

20 young adult worms of each strain were transferred from an RNA*i* feeding plate onto a well on the aldicarb plates, using a platinum rod under the light microscope. 20 more young adult worms were transferred onto a well adjacent. A total of 40 worms, 20 per well, were transferred from the feeding plates for each strain, as shown in the schematic diagram below. Each worm placed on the aldicarb plates were touched with the tip of the platinum rod every 30 minutes to test check for the touch response for the total duration of 3 hours. The number of worms per well that responded to touch every 30 minutes was recorded. The experiment was carried out in triplicates.

A Schematic diagram to show the arrangement of 40 worms of each strain on an aldicarb plate:



6.4.5 Statistical analysis for the Aldicarb assay

(This part of the project was carried out by Mr. Giovanni D'Ario, IFOM, Milan, Italy)

The data collected from the aldicarb assay as described in the previous section 6.3.4 were subjected to a statistical analysis in order to represent the significance of the genetic interactions. The statistical significance was shown by a method described below, which utilized the R statistical software [137].

6.4.5.1 Description of the Data Set

The raw data set was divided into four files, one for each of the genetic backgrounds: *ehs-1, itsn-1, rme-1, reps-1.* In each of these files the following information was recorded:

1) block: an accessory variable used to indicate the particular comparison between a genetic background and a gene, which was knocked down. Such a combination represents the experimental unit block, hence the name.

2) replicate: a number indicating the particular replicate within the same type of

comparison. The number of these replicates is not constant across experimental blocks and ranges from 2 to 6.

3) gene: a categorical variable used to indicate either the silenced gene or the control group within each class of comparison.

4) strain: a categorical variable indicating the genetic background, including the wild type group.

5) time: the time at which the animals were stimulated and the number of reacting animals recorded. Time zero is the time at which aldicarb was administered. Measurements were taken at 0.5, 1, 1.5, 2, 2.5, 3 hours.

Despite the presence of 6 time points, all the animals were uncoordinated (with only one exception) at 2.5 hours, therefore there are only four informative time points. A further complication is the fact that the initial number of worms is not constant. On the other hand, there are no cases of censored survival times, therefore we decided to pursue a simple strategy focused on the individual (approximate) survival times rather than on the counts of worms still reacting at the tactile stimulus at each time point.

For each combination of the factors within each of the four genetic backgrounds, we operated as described in the example shown below:

Example 1: The first replicate of the combination of the *ehs-1* genetic background and the silenced gene *lin*-10.

There were 60 worms at time 0 (T=0), 53 at T = 0.5, 20 at T = 1.0, 9 at T = 1.5 hours, and 0 worms at T = 2.0 onwards. We reorganized these counts creating a vector of 60 elements, each containing the approximate survival time of a worm. In this case the vector would have 9 elements containing a survival time of 2.0 hours, 11 of 1.5 hours,

33 of 1.0 hour and 7 of 0.5 hour. An approximate survival time can be calculated as T = 1.5 hours, even though the worms became uncoordinated at an unknown time in the interval having T = 1.0 and T = 1.5 hours as endpoints. This uncertainty is compensated by the fact that every worm now brings a piece of information, increasing the statistical power of the analysis described below.

6.4.5.2 Statistical Analysis

B indicates the genetic background of worms that has been impaired. B can take values *ehs-1, rme-1, itsn-1, reps-1.*

G indicates an impaired gene, where G can be *lin-10, alx-1, dab-1* etc.

N indicates the wild type phenotype.

B+G represents the combined impairment of both B and G.

Depending on the reciprocal relationships among these factors, we can have several possible scenarios. We defined the *genetic interaction* as the situation where the B+G phenotype differs from both B and G alone.

It is advantageous to consider the difference between the survival time of a particular factor (using the generic term "factor" to indicate B, G, or B + G) and the wild type condition, rather than the absolute survival times.

We therefore introduce the quantities:

$G = G\_N$; $B = B\_N$; $\_\_B\ G\_ = \_B\_\ G\_\ N$

G_N indicates the application of the knockdown genes (G) in the wildtype background (N). Whereas B_N indicates the effect of the genetic background of the *eh* mutants in the wildtype genetic background (N). The factor that contains the combination of the previous two factors, the effect of knockdown of genes (G) and that of the mutant

genetic background (B) is represented by _ _ *B G*_, which is the combined effect of B and G in the genetic background of wild type (N). This notation allows us to simplify greatly the number of possible scenarios and to formalize our definition of genetic interaction.

The first condition to be tested for is:

_ _ B_G_ = _B.

If this identity holds, there can be no genetic interaction, since it simply states that the phenotype of the doubly impaired class does not differ from that of the background-impaired one. This equality represents a sufficient but not necessary condition for the absence of genetic interaction. Provided that the condition _ _B _ G_ ≠ _B holds, the identity _ _ B_G_ = _G is then tested. If the latter condition is true, even though it appears to be different from the background phenotype, the doubly impaired phenotype is not significantly different from the G phenotype. This suggests that the observed difference compared to the wild type is due to G alone, rather than to a combined effect of B and G. In this case, therefore, the presence of genetic interaction can be excluded.

In conclusion, the two conditions for having a genetic interaction are _ _B_G_≠_B and _ _B_G_≠_G. It is important to note that equality and inequality must be looked at in the context of a statistical inferential procedure and not in a strict algebraic sense. For this reason, a condition that is expressed as _ _B_G_≠_B means that the null hypothesis that these two quantities are equal is rejected, with a given level of significance level.

The scenarios discussed so far do not exclude all the possibilities, and we can further refine our analysis including other possibilities. In Figure I, a schematic representation of all the situations we have taken into account is depicted.

6.4.5.3 Linear Models

To identify the combination of experimental factors giving rise to a genetic interaction we used the following linear model:

$t_i = \_0\_ \ _{12}U_2 \_13U_3 \ \_ \ _{14}U_4\_\_$

where t_i is the survival time for the i^{th} worm, $\__i$ is a random error with zero mean and variance $\_^2$. An experiment, in which *lin-10* was knocked down (k.d.) in the wild type *N2* and *ehs*-1 mutants, will be used as an example. Within the experimental block, the following combinations of factors can be found:

1) wild type *ehs-1* / wild type *lin-10.*

2) k.d. *ehs-1* / wild type *lin-10.*

3) wild type *ehs-1*/ k.d. *lin-10.*

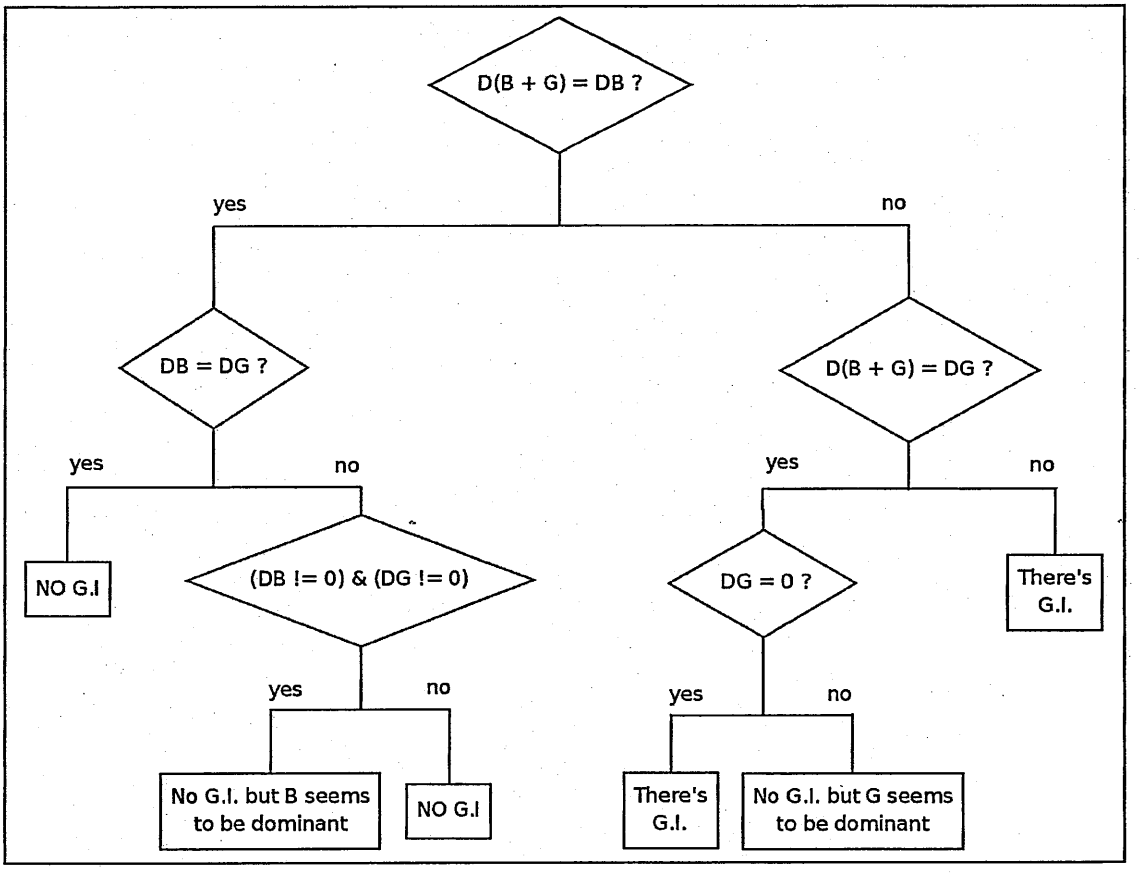
4) k.d. *ehs-1* / k.d. *lin-10.*

The average survival time for the worms belonging to group 1 is given by coefficient $-_0$, which is the reference against which factors such as _B, _G are computed. The coefficients $-_{1k}$ represent the change in the average survival time for the worms in the k^{th} group, for k = 2,3,4 (group 1 is accounted for by $-_0$). U_k is a "dummy variable", which assumes a value of 1 if the worm belongs to the k^{th}, or assumes a value of zero otherwise.

Once a model is fitted we obtain an estimate of the four parameters, and the associated standard error. Thus, the comparisons described above can be performed and a p-value for every single combination is obtained.

In this analysis, the averaged survival times were arranged across the replicates of the same experimental block. All the tests were two-sided.

Figure I. A Tree representation of the algorithmic procedure we have followed to establish the presence or absence of genetic interaction.



6.4.6 Egg laying assay

24 adult worms of *N2* and *rme-1* (*b1045*) strain that were subjected to RNA*i* by feeding, as described in Section 6.3.3, were used for this assay. After 4 days of incubation at 15°C, an L4-staged worm was transferred onto to a small NGM agar well (in a 12-well plate) prepared with lawn of *E.Coli OP50*. For each knockdown experiment in *N2* and in *rme-1* background, a total of 24 L4-staged worms were transferred to individual well. The worms were continuously transferred into a new well every 36 hours for a total period of 5-7 days, until each individual worm had stopped laying eggs. The worms were incubated at 15°C constantly, apart from when they were being transferred into new wells at room temperature. The total number of eggs laid by each worm was counted. The total egg counts of three worms were not included when calculating the

average, because of an early death after 1 day, or due to two worms having extremely low brood sizes of 2-3 eggs in the total period of 7 days. The average for each knockdown experiment was calculated from the duplicated experiments with 21 worms.

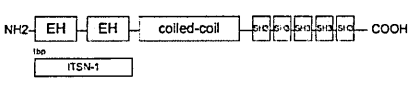
III. RESULTS

7. Y2H Screenings

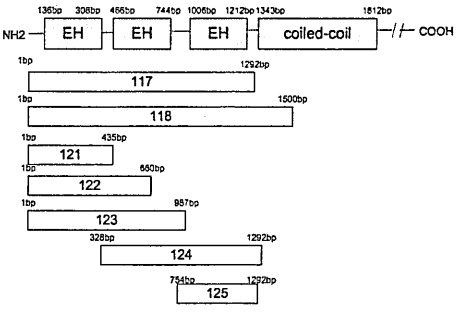
The EH domains of five *C. elegans* EH domain-containing proteins (R10E11.6, ITSN-1, EHS-1, REPS-1 and RME-1) were cloned as baits (Figure 1) to screen against the cDNA library of *C. elegans*, prepared from a mixed population of all developmental stages.

Figure 1 The Schematic diagrams to show the regions cloned as baits for the Y2H screening of ITSN-1, EHS-1, RME-1, REPS-1, and R10E11.6

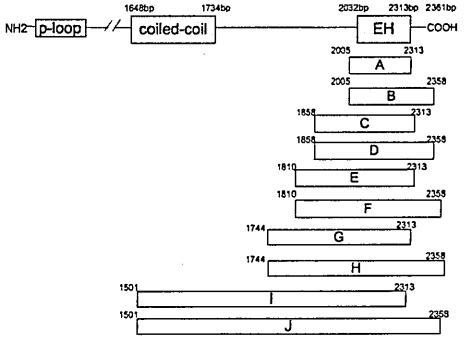
a. ITSN-1



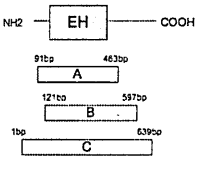
b. EHS-1



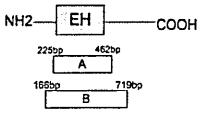
c. RME-1



d. REPS-1



e. R10E11.6



The EH domain-containing regions were cloned in-frame with the GAL4DBD, whereas the cDNAs were cloned in-frame with the GAL4AD. The expression of the chimera proteins of GAL4DBD and the EH domain-containing regions were checked by Western Blots using anti-GAL4DBD antibody (Figure 2).

Figure 2 Western blots with Anti-GAL4DBD to show the expression of the bait constructs in-frame with GAL4 DBD.

a. REPS1 C, RME-1 Bait B, ITSN-1, EHS-1 123, EHS-1 125

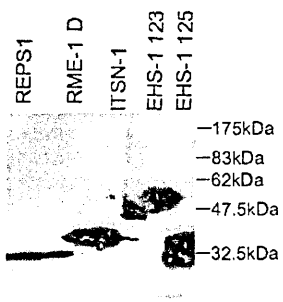


Fig.2a The expected molecular weights of the Reps-1 bait, one of the RME-1 baits, ITSN-1 bait, and two EHS-1 baits :
Reps-1 C: 40.9kDa
RME-1 D: 34.1kDa
ITSN-1: 47.0kDa
EHS-1 123: 53.0kDa
EHS-1 125: 30.9kDa
The band corresponding to RME-1 bait D was repeatedly detected around 37-40kDa, higher than expected. In comparison, the band corresponding to REPS-1 bait ran lower than expected, near 35kDa. The correct DNA sequences of all bait constructs were confirmed.

b. RME-1 constructs D, F and H

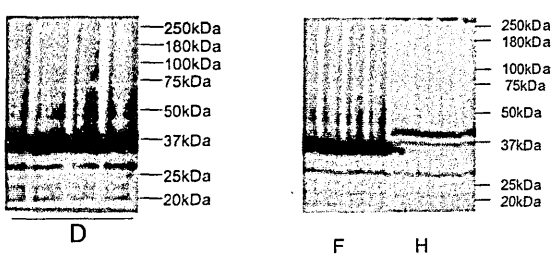


Fig.2b The expected molecular weights of the RME-1 baits used in Y2H screenings:
D: 34.1kDa
F: 35.8kDa
H: 38.8kDa
The band corresponding to the bait D was detected at a molecular weight higher than expected, closer to 37kDa, as described also above in Figure 2a.

c. R10E11.6 bait

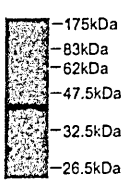


Figure 2c. The expected molecular weight of R10E11.6 bait :
R10E11.6 B: 35.6kDa

The cDNA library and the baits were co-transformed in *Mav203* yeast strain, which has three reporter genes (*URA3*, *HIS3 and LacZ*) with the GAL4 promoter regions in the genome, as described in Section 3.2. In order to ensure that the expression of the bait

alone does not self-activate the reporter genes, the clones transformed with the bait and the empty prey vector were tested by X-gal assays (Figure 3 and 4).

In addition, *Mav203* cells express a basal level of *HIS3*, which can be titrated out in the presence of 3 -Amino-1,2,4-Triazole (3AT). Therefore the minimum concentration of 3AT required to sensitize the selection by *HIS3* was determined for the clones transformed with the bait and the empty prey vector (Figure 5).

Figure 3 X-gal assays of the co-transformants of each bait construct (pDBLeu-Bait) with an empty prey vector (pPC86) to ensure that the baits used for screenings did not self-activate The expression of the reporter gene, LacZ.

A,B,C,D,E: Yeast Control strains transformed with plasmids containing no inserts (A), or transformed with plasmids expressing interactors of increasing interaction strengths in the order of B-E, as described in detail in Table 1 in Section 6.2.3.

1,2,3,4,5: Five different co-transformants tested in order to ensure that the self-activation was not observed not only in one clone but in multiple clones.

The co-transformants of the EHS-1 bait 123 and pPC86 were tested alongside five co-transformants of EHS-1 bait 117, which contains all three EH domains and had shown self-activation of *LacZ* when tested previously (Refer to the later section 7.2.1.)

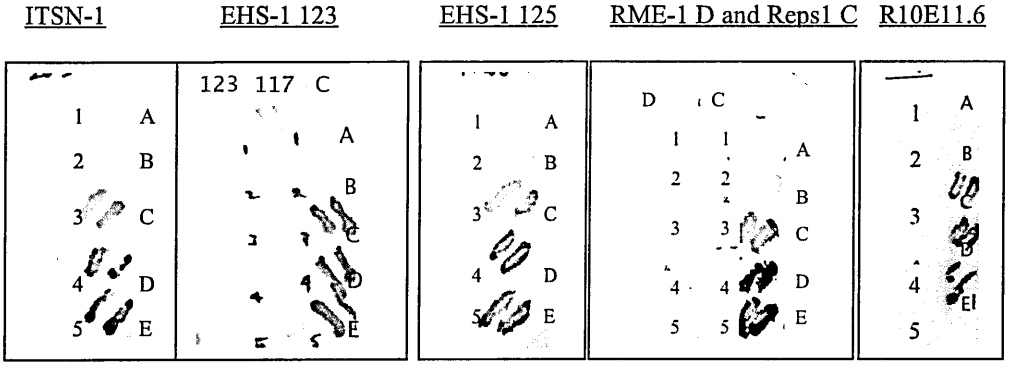


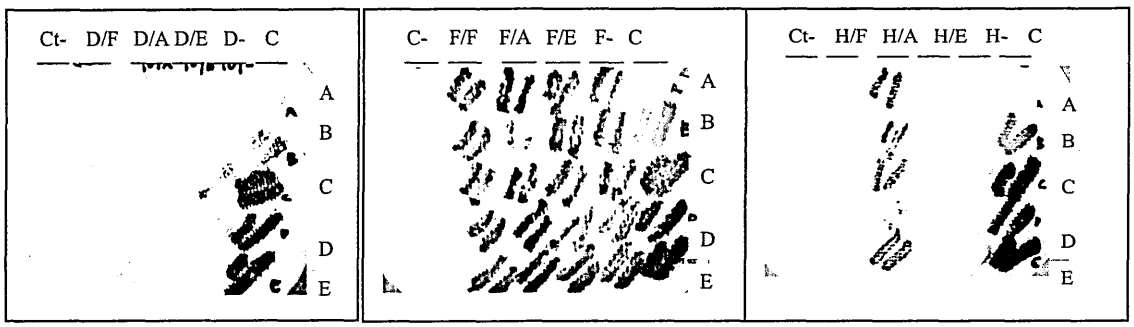
Figure 4 X-gal assays of the various RME-1 bait constructs D, re-transformed with an empty vector pPC86 (C-) or with the cDNA encoding epn-1 (E), alx-1(A) and F15C11.2 (F) in-fusion with GAL4AD, alongside a negative control, Reps-1 C/pPC86 (Ct-).

Five co-transformants were tested to ensure that the self-activation was not observed not only in one clone but in multiple clones.

The co-transformants shown in this figure were transformed with the plasmids as follows

Ct-: pDBLeu-REPS1/ pPC86 with noinsert	Ct-: pDBLeu-REPS1/ pPC86 with noinsert	Ct-: pDBLeu-REPS1/ pPC86 with noinsert
D/F: pDBLeu-BaitD/ pPC86-F15C11.2	F/F: pDBLeu-BaitF/ pPC86-F15C11.2	H/F: pDBLeu-BaitH/ pPC86-F15C11.2
D/A: pDBLeu-BaitD/ pPC86-alx-1	F/A: pDBLeu-BaitF/ pPC86-alx-1	H/A: pDBLeu-BaitH/ pPC86-alx-1
D/E: pDBLeu-BaitD/ pPC86-epn-1	F/E: pDBLeu-BaitF/ pPC86-epn-1	H/E: pDBLeu-BaitH/ pPC86-epn-1
D-: pDBLeu-BaitD/ pPC86 with no insert	F-: pDBLeu-BaitD/ pPC86 with no insert	H-: pDBLeu-BaitH/ pPC86 with no insert
C: Yeast control strains A-E	C: Yeast control strains A-E	C: Yeast control strains A-E

a. Bait D **b. Bait F** **c. Bait H**

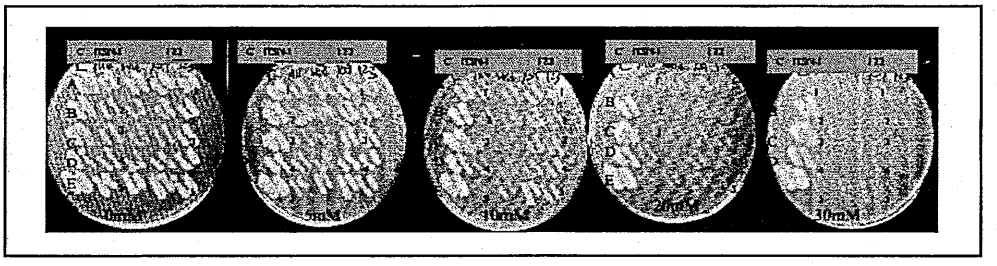


The co-transformants of the cDNA library and the baits were tested initially for the expression of *HIS3* in the presence of 3AT, at a concentration that was set up prior to the screenings. The clones that were positive for *HIS3* expression were further tested for the expression of *LacZ* by X-gal assays, and of *URA3* by replica plating onto minimum medium lacking uracil.

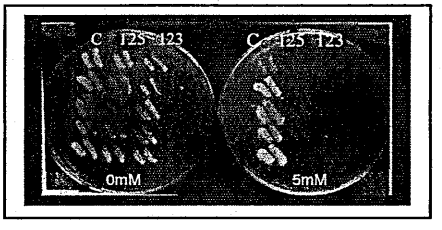
Figure 5 The replica plates of pDBLeu-bait/pPC86 transformants onto plates containing increasing concentration of 3AT from 0mM to 100mM (SC –Leu, -Trp, -His +3AT) in assays to determine the concentration of 3AT that is required titrate out the basal HIS3 expression by the co-transformants of every bait.

A,B,C,D,E: Yeast Control strains transformed with plasmids containing no inserts (A), or transformed with plasmids expressing interactors of increasing interaction strengths in the order of B-E, as described in detail in Table 1 in Section 6.2.3.

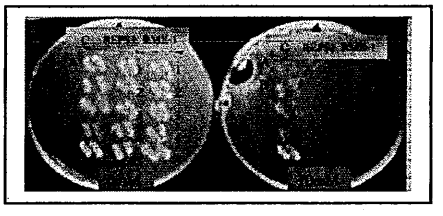
a. pDBLeu-ITSN-1/pPC86 and pDBLeu-EHS-1 123/pPC86 co-transformants



b. pDBLeu-EHS-1 125/pPC86 co-transformants



c. pDBLeu-Reps-1 C/pPc86 and pDBLeu-RME-1 D/pPC86 co-transformants



d. pDBLeu-R10E11.6/pPC86 co-transformants

In all tests used to monitor the expression of the reporter genes, the control strains A, B, C, D and E, provided from the Invitrogen were used as reference. The control strains express interactors that interact in an increasing order from A to E. The interactors expressed by each control strain are described in detail in Table 1, under the section

6.2.3. The control strain A was used as negative control, whereas the control strains B, C, D, and E were used as the positive controls.

No interactors were found for the putative EH protein, R10E11.6. On the other hand, the Y2H screenings identified a total of 26 proteins as the interactors of ITSN-1, EHS-1, REPS-1, and RME-1 in *C. elegans*, including several proteins, which are common interactors between two or three of the EH proteins.

7.1 ITSN-1 Y2H Screenings

7.1.1 Preparation of ITSN-1 bait

The EH domains present in the N-terminal of ITSN-1 were cloned in-frame with GAL4DBD in a vector pDBLeu as the bait, as shown in Figure 1a. It was ensured prior to the screening that the bait was expressed in-frame with GAL4DBD by Western blot with anti-GAL4DBD antibody on the protein precipitates of the yeast culture transformed with the bait constructs and the empty prey vector. The expression of the fusion protein GAL4DBD-ITSN-1 is shown by the band, which is specifically recognized by an anti-GAL4DBD antibody in the Western Blot, at its expected molecular weight of 47.0kDa (Figure 2a).

Five colonies from the same co-transformation were used in X-gal assay in order to ensure that the bait is not able to self-activate the reporter gene *LacZ*. The clones expressing the ITSN-1 bait together with GAL4AD expressed by the empty prey vector pPC86, were shown to be negative for *LacZ* expression. The minimum concentration of 3AT required to titrate out the basal expression of another reporter gene *HIS3* for the yeast clone expressing the ITSN-1 bait was determined to be 10mM, as shown in Figure 5a.

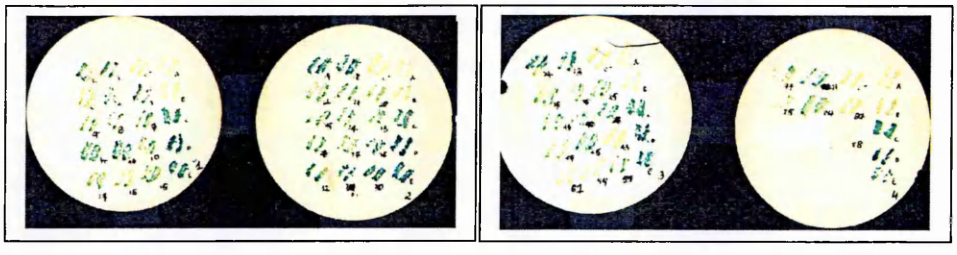
7.1.2 Y2H screenings with ITSN-1 (I) & (II)

A total of two screenings were carried out using the bait containing the EH domain of ITSN-1. In the first screening (I), 88 clones were positive for the initial selection for the expression of *HIS3* in the presence of 10mM 3AT. 43 out of these 88 clones were positive for the expression of *LacZ* gene (Figure 6a) whereas 25 clones were positive for the *URA3* expression (results not shown). The expression of the three reporter genes by each clone is summarized in Table 3.

Figure 6 X-gal Assay to test the expression of **LacZ** *reporter genes by the clones fished for ITSN-1 Y2H screenings*

A,B,C,D,E: Yeast Control strains transformed with plasmids containing no inserts (A), or transformed with plasmids expressing interactors of increasing interaction strengths in the order of B-E, as described in detail in Table 1 in Section 6.2.3.

a. ITSN-1 Y2H screening I: X-gal assays of a total of 48 clones that were identified as positive for the expression of *HIS3* with 10mM 3AT. The 48 clones out of the 88 clones were tested to be positive for growth in –HIS medium when the single colonies were streaked on a plate lacking HIS. The number of the clones in the assay refers to the initial clone number out of the 88 clones.



b. **ITSN-1 Y2H Screening II: X-gal assays of a total of 100 clones that were identified as positive for the expression of *HIS3* with 10mM 3AT.**

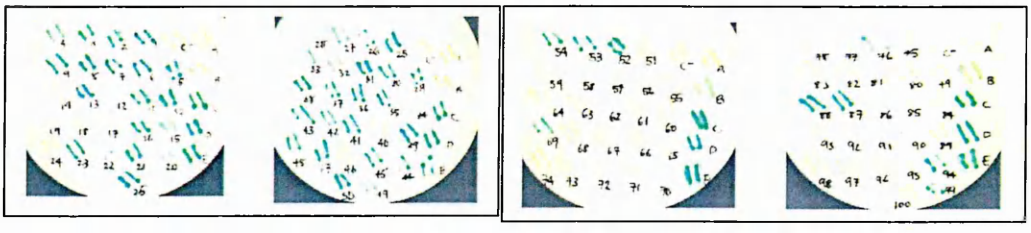


Table 3 The expression of the reporter genes, LacZ and URA3, by the ITSN-1 interacting-clones, which were initially selected for the expression of HIS3 in the presence of 10mM 3AT

The levels of expression were compared with that of the control strains A-E as follows: A:-, B: +, C: ++, D:+++, E: ++++. Note that the intensity shown in the figure may not correspond exactly to that observed during the experiment as the intensity of the colour changed during storage of the membrane after the experiment.

(Clones # 4, #12-13, #42, #44-45, #48-54, #56-58, #62-70, #72-76, #78-79, #81-84, and #86-87 were omitted as they were contaminants.)

Clone #	*LacZ*	*URA3*	*HIS3*
1	+++	+	++
2	++	+	++
3	++	-	++
5	+	-	++
6	++	-	++
7	+++	+	++
9	++	++	++
10	++	+	++
11	+	+	++
14	++	-	++
15	+	-	++
16	+	-	++
17	+	-	++
18	+++	++	++
19	+++	++	++
20	+	-	++
21	++	+	++
22	++	++	++
23	++	+	++
24	++	+	++
25	++	+	++
26	++	+	++
27	++	++	++
28	++	+	++
30	+++	-	++
31	++	+	++
32	++	+++	++
33	+++	-	++
34	++	+	++
35	++	-	++
36	++	-	++
37	++	-	++
38	++	-	++
40	++	+	++

Clone #	*LacZ*	*URA3*	*HIS3*
41	++	+++	++
43	-	+	++
46	++	-	++
47	++	+/-	++
55	++	-	++
59	-	-	++
60	-	-	++
61	-	-	++
71	++	-	++
77	++	-	++
80	++	-	++
85	+	-	++
88	-	-	++

In the second screening (II), 100 clones were selected for *HIS3* expression in the presence of 10mM 3AT. As shown in Table 4, 52 out of these 100 clones were shown to express *LacZ* by the X-gal assay as shown Figure 6b. In comparison, 32 out of these 100 clones were positive for the expression of *URA3*, and 22 clones, which showed a very weak expression of URA3 (indicated as +/- in Table 4). The expression of the reporter genes *LacZ*, *URA3* and *HIS3* are shown in Table 4.

Table 4 The expression of the reporter genes,* LacZ *and* URA3, *by the ITSN-1 interacting-clones, which were initially selected for the expression of* HIS3 *in the presence of 10mM 3AT.

The levels of expression were compared with that of the control strains A-E as follows: A:-, B: +, C: ++, D:+++, E: ++++. Note that the intensity shown in the figure may not correspond exactly to that observed during the experiment as the intensity of the colour changed during storage of the membrane after the experiment.

Clone#	*LacZ*	*URA3*	*HIS3*
1	+	-	++
2	+++	-	++
3	+	++	++
5	++	-	++
6	++	-	++
7	+++	-	++
8	++	+	++
9	++	-	++
10	++	+++	++
11	++	-	++
12	-	+/-	++
13	++	+/-	++
14	+	+++	++
15	+	-	++
16	++	-	++
17	-	-	++
18	-	-	++
19	-	-	++
20	+	-	++
21	++	++	++
22	+	-	++
23	++	++	++
24	-	-	++
25	+++	-	++
26	++	-	++
27	+	+	++
28	-	+	++
29	++	++	++
30	++	-	++
31	+++	-	++
32	+	-	++
33	+	-	++
34	-	-	++
35	++	+	++
36	++	+	++
37	++	-	++
38	+++	-	++
39	+++	-	++
40	-	-	++
41	+++	+/-	++
42	++	+/-	++
43	++	+/-	++
44	++	-	++
45	++	-	++
46	-	+	++
47	++	+/-	++
48	++	+/-	++
49	+	-	++
50	++	+/-	++
50	++	+/-	++
51	-	+/-	++
52	+++	+/-	++
53	++	-	++
54	++	+/-	++
55	-	+/-	++
56	-	+/-	++
57	-	+/-	++
58	-	-	++
59	-	-	++
60	-	+	++
61	-	+/-	++
62	-	+/-	++
63	-	-	++
64	-	+/-	++
65	-	+	++
66	-	+/-	++
67	-	+	++
68	-	-	++
69	++	+	++
70	-	+/-	++
71	-	+/-	++
72	-	-	++
73	-	-	++
74	-	+	++
75	-	+	++
76	+	+	++
77	-	-	++
78	-	-	++
79	-	+	++
80	+	+	++
81	-	+	++
82	-	-	++
83	-	-	++
84	-	+	++
85	-	+	++
86	-	+	++
87	++	-	++
88	++	-	++
89	-	+	++
90	+	+	++
91	-	+	++
92	-	-	++
93	-	+/-	++
94	++	+	++
95	-	+	++
96	-	+	++
97	-	-	++
98	-	-	++
99	++	-	++
100	-	+	++

7.1.3 Interactors of ITSN-1

A total of 95 clones were positive for the expression of *LacZ* and/or *URA3*. Subsequent sequencing of the cDNA library insert of these 95 clones identified a total of 16 distinct putative interactors that were translated in-frame with the GAL4AD. The DNA sequences of the positive clones were analysed to determine whether the cDNA inserts is in-frame with the GAL4AD, as described in Materials and Method. Following the re-transformation assays, 10 proteins were confirmed as putative interactors of ITSN-1, namely: EPN-1, LIN-10, PQN-32, TFG-1, T05E7.4, F46H5.7, F15C11.2, DAB-1, K04H5.2 and BE0003N10.3 (Figure 7).

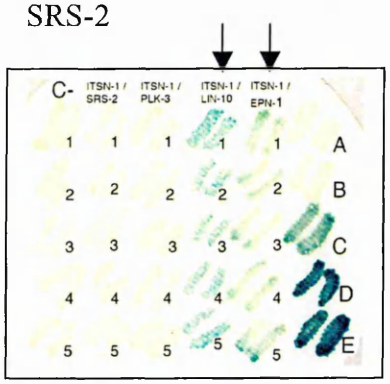
Figure 7. The X-gal Assays for the Re-transformation assay of the putative positive interactors that were identified from the Y2H screenings with ITSN-1.

A,B,C,D,E: Yeast Control strains transformed with plasmids containing no inserts (A), or transformed with plasmids expressing interactors of increasing interaction strengths in the order of B-E, as described in detail in Table 1 in Section 6.2.3.

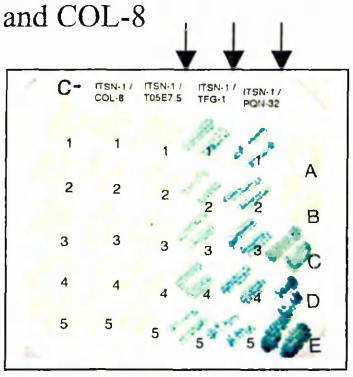
1,2,3,4,5: Five different co-transformants tested in order to ensure that the *LacZ* activation was not observed not only in one clone but in multiple clones.

The positive interactions detected in the X-gal assays are marked with arrows.

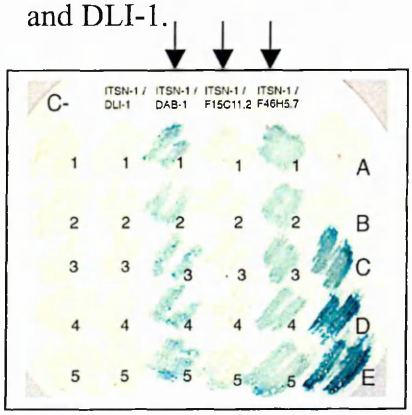
1) EPN-1, LIN-10, PLK-3, and SRS-2



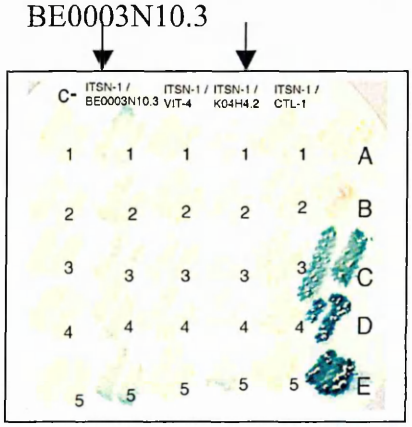
2) PQN-32, TFG-1, T05E7.5, and COL-8



3) F46H5.7, F15C11.2, DAB-1, and DLI-1.



4) CTL-1, K04H4.2, VIT-4, and BE0003N10.3



7.2 EHS-1 Y2H Screenings

7.2.1 Preparation of EHS-1 baits

Two baits containing all three EH domains present in the N-terminal of EHS-1, indicated as constructs 117 and118 in Figure 1b, were able to self-activate *LacZ* expression (construct 117 shown in Figure 3b). In order to avoid using baits that self-activate the reporter genes, several baits were constructed that separate the three EH domains. Bait construct 121 contained only the first EH domain, whereas Bait 122 was designed to contain the first EH domain and the half of the second EH. Bait 122 was constructed in order to include the surrounding region of the first EH domain, but not including the second EH domain, which starts 54 amino acids after the first EH domain, in case two EH domains together caused self-activation. Bait 123 containing both the first and the second EH domain, showed no self-activation (Figure 3b). When the third EH was expressed on its own (Bait 125), the ability to self-activate *LacZ* was not observed (Figure 3c), whereas Bait 124 containing the second and the third EH domains showed self-activation (Figure 3b).

Bait 123 was chosen for its complete representation of the region containing the first two EH domains. The bait 125 was then used in additional Y2H screenings to compensate for any interactions that may be mediated specifically by the third EH domain. The expression of Bait 123 and Bait 125 in-frame with GAL4DBD were detected by anti-GAL4DBD Western Blot as the bands corresponding with the expected molecular weight, 53.0kDa and 30.9kDa, respectively (Figure 2a). The minimum concentrations of 3AT appropriate for titration of *HIS3* basal expression with the bait 123 was determined to be between 20mM and 30mM (Figure 5a). In comparison, the appropriate concentration of 3AT for Bait125 was determined as 10mM (Figure 5b).

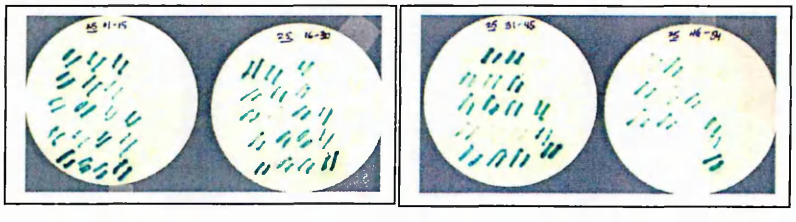
7.2.2 Y2H Screenings with EHS-1 123 (I) & (II)

Two screenings with the bait 123 were performed, one in the presence of 25mM 3AT, and another in less stringent conditions, using 10mM 3AT. In the Y2H screening with bait 123 (I), 54 clones were selected for the expression of *HIS3* in the presence of 25mM 3AT. 51 out of these 54 clones expressed *LacZ* as assessed by the X-gal assay (Figure 8a). X-gal assays were repeated to ensure reproducibility of results. Indeed, there was no discrepancy except for clone number 42, which was positive for the first X-gal assay but was negative in the second X-gal assay, although differences in the level of activation was observed in several clones (Table 5). In comparison, 49 out of these 54 clones were positive for the expression of *URA3*.

Figure 8 X-gal Assay to test the expression of LacZ reporter gene by the clones fished for EHS-1 Y2H screenings.

A,B,C,D,E: Yeast Control strains transformed with plasmids containing no inserts (A), or transformed with plasmids expressing interactors of increasing interaction strengths in the order of B-E, as described in detail in Table 1 in Section 6.2.3.

a. EHS-1 123 Y2H screening I: X-gal assays of a total of 54 clones that were identified as positive for the expression of *HIS3* with 25mM [3AT].



b. EHS-1 123 Y2H screening II: X-gal assays of a total of 110 clones that were identified as positive for the expression of *HIS3* with 10mM [3AT].

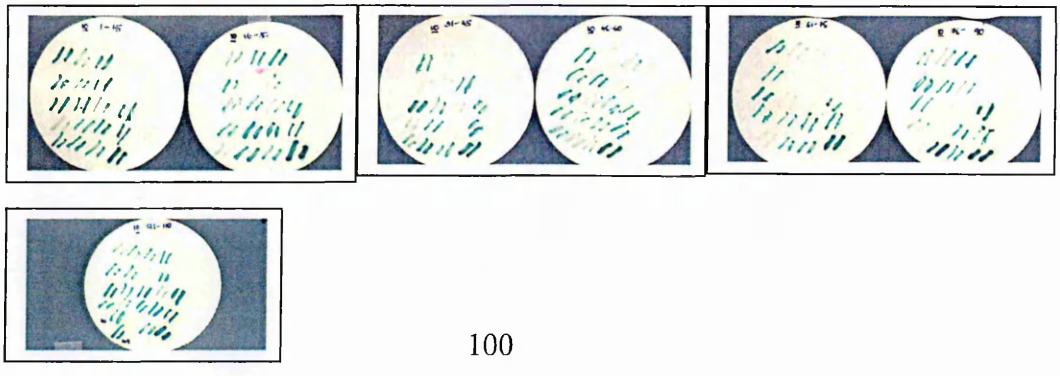


Table 5 The expression of the reporter genes, **LacZ** *and* **URA3**, *by the EHS-1-interacting clones, which were initially selected for the expression of* **HIS3** *in the presence of 25mM 3AT.*

The levels of expression were compared with that of the control strains A-E as follows: A:-, B: +, C: ++, D:+++, E: ++++. Note that the intensity shown in the figure may not correspond exactly to that observed during the experiment as the intensity of the colour changed during storage of the membrane after the experiment.

Clone #	*LacZ*		*URA3*	*HIS3*
	1	2		
1	+++	+++	+++	+++
2	++	++	+++	+++
3	+++	+++	++	+
4	++	+	++	+
5	+++	+++	+	+++
6	+++	+++	+	++
7	+	+	++	+
8	++	++	++	++
9	++	++	++	+
10	+++	+++	+	++
11	++	+	++	+
12	+	++	++	++
13	++	++	+	++
14	+++	++	++	+
15	+++	+++	++	+++
16	+	++	-	+++
17	++	++	+++	++
18	+++	+++	+++	+++
19	+++	+	+	+
20	+++	++	+++	++
21	++	++	++	+++
22	+++	++	+++	+++
23	++	+/-	+	+
24	++	++	++	++
25	+++	++	+++	++
26	+++	++	++	++
27	++	++	+	+
28	++	++	++	+
29	+++	++	++	+
30	+++	+++	+	++
31	++	++	+	+++
32	+++	+++	++	+++
33	-	-	-	++
34	+++	++	++	+++
35	+	+	+	+
36	+	+	++	++
37	+	++	++	+++
38	++	+++	++	+++
39	+	+	+	+
40	+/-	+/-	+/-	+
41	-	-	+++	-
42	-	+	+	+
43	++	+++	++	++
44	+++	+++	+++	++
45	+	++	+	+
46	-	-	-	-
47	+	++	+	+/-
48	-	+/-	-	+
49	++	++	+	++
50	+	+/-	+/-	+
51	+	++	-	+++
52	-	-	+/-	-
53	+++	++	+++	+
54	++	++	+++	+/-

In the second less stringent screening with bait 123 (II) in the presence of 10mM of 3AT, 110 clones were positive for the initial selection of *HIS3* expression. 102 out of these 110 clones were also positive for the expression of *LacZ* gene for at least one of the duplicated X-gal assays (Figure 8b), whereas 96 clones were positive for the *URA3* expression (results not shown). The expression of the three reporter genes: *LacZ*, *URA3* and *HIS3*, by the individual clones are summarized in Table 6.

Table 6 The expression of the reporter genes,* LacZ *and* URA3, *by the EHS-1 interacting-clones, which were initially selected for the expression of* HIS3 *in the presence of 10mM 3AT in EHS-1 Y2H.

The levels of expression were compared with that of the control strains A-E as follows: A:-, B: +, C: ++, D:+++, E: ++++. Note that the intensity shown in the figure may not correspond exactly to that observed during the experiment as the intensity of the colour changed during storage of the membrane after the experiment.

Clone #	*LacZ*		*URA3*	*HIS3*
	1	2		
1	++	++	+++	++
2	+	+	+	+
3	+++	+++	++	+++
4	+++	+++	+++	++
5	++	+	++	+/-
6	++	+	++	+/-
7	+	+	++	+/-
8	+++	++	+++	++
9	+++	+++	+	++
10	++	+++	++	+
11	++	+	+	+
12	+	-	++	+/-
13	+	+	-	++
14	+++	++	+++	+
15	++	+++	++	+
16	++	+++	+	+++
17	++	++	+++	+
18	++	+	++	+/-
19	++	+	++	+/-
20	+	+/-	++	+/-
21	+	++	++	++
22	+	+	+	+
23	+++	++	++	+++
24	+	+	++	++
25	+++	+++	++	++
26	+++	+++	+++	+++
27	+	++	+	++
28	+	+	+++	+++
29	++	++	+	+
30	+	+/-	-	-
31	-	-	-	-
32	++	+/-	+	+
33	+++	++	++	++
34	-	++	++	++
35	-	-	+	+
36	+++	-	+	+
37	-	+/-	+	-
38	+	+	+++	+++
39	++	+++	+++	+++
40	++	-	+	+
41	+	++	+	++
42	+	+	++	+
43	++	++	+++	++
44	+	+++	+++	+++
45	-*	++	+	-
46	+++	+	+	+
47	++	+	+	++
48	++	++	++	++
49	++	+	+	-
50	++	++	+	+
51	++	++	+	+
52	-	++	+	+++
53	-	+	+	+
54	++	++	+	+
55	++	++	+	+++
56	-	+	++	++
57	-	++	++	+++
58	+	+	++	++
59	+++	+	+++	+
60	-*	++	+	+
61	-	-	-	+
62	+	+	+++	+/-
63	++	++	+++	+++
64	+++	-	-	+
65	+	-	-	-
66	-	++	+/-	++
67	+	-	-	+/-
68	+	+/-	+	+
69	+++	+++	+	+++
70	-	+++	++	++
71	++	+	+	+
72	+	+++	-	-
73	++	++	+++	+
74	+++	++	+/-	-
75	+	+/-	+	+
76	++	+++	+++	+++
77	-	++	++	+++
78	+++	++	++	++
79	+	++	++	+++
80	++	++	+++	+++
81	+	+++	+++	+++
82	-	-	+	-
83	+	-	+	+
84	+++	++	+++	+++
85	+	+++	+++	++
86	+	-	-	-
87	++	+	-	+
88	-	++	+++	+++
89	+++	+++	++	+++
90	-	-	-	-
91	+++	+++	+/-	++
92	+++	++	++	++
93	++	++	++	++
94	++	+	+	++
95	++	+++	+	+/-
96	-	-	-	+
97	++	++	+++	+
98	++	++	+++	++
99	++	+	+++	+
100	+++	+++	+++	++
101	++	++	+++	+++
102	+	+++	++	+++
103	++	+++	++	++
104	-	+++	+++	+
105	+	+	+	+
106	+	+	++	+
107	++	++	++	++
108	-	-	-	+
109	+++	+++	-	++
110	++	++	+++	++

7.2.3 Y2H Screenings with EHS-1 125 (I) & (II)

The first Y2H screening with bait 125 resulted in 50 clones, which were found to be positive for *HIS3* selection in the presence of 5mM 3AT. 7 out of these 50 clones were shown to express *LacZ* by the X-gal assay, as shown in Figure 9a. In comparison, 4 out of these 50 clones were positive for the expression of *URA3*, which appeared to show a level of growth slightly higher than the negative controls, the bait alone and control strain A (results not shown). The expression of the three reporter genes: *LacZ*, *URA3* and *HIS3*, by the individual clones are summarized in Table 7.

In the second more stringent screening with bait 125 in the presence of 10mM of 3AT, 25 clones were positive for the initial selection for the expression of *HIS3*. 13 out of these clones were positive for the expression of *LacZ* gene (Figure 9b) whereas only 3 clones were positive for the *URA3* expression (results not shown). The expression of the three reporter genes: *LacZ*, *URA3* and *HIS3*, by the individual clones are summarized in a table in Table 8.

Table 7 The expression of the reporter genes,* LacZ *and* URA3, *by the EHS-1 interacting-clones that were initially selected for the expression of* HIS3 *in the presence of 5mM 3AT.

The levels of expression were compared with that of the control strains A-E as follows: A:-, B: +, C: ++, D:+++, E: ++++. Note that the intensity shown in the figure may not correspond exactly to that observed during the experiment as the intensity of the colour changed during storage of the membrane after the experiment.

Clone #	*LacZ*	*URA3*	*HIS3*
1	-	+	+
2	-	+	+
3	-	+	+
4	++	-	+
5	-	++	+
6	+	+	+
7	-	-	+
8	-	-	+
9	-	-	+
10	-	-	+
11	-	-	+
12	-	-	+
13	-	-	+
14	-	-	+
15	-	-	+
16	-	-	+
17	-	-	+
18	-	-	+
19	-	-	+
20	-	-	+
21	+	++	+
22	-	-	+
23	-	-	+
24	-	-	+
25	-	-	+
26	-	-	+
27	+	-	+
28	+	-	+
29	+	-	+
30	-	+/-	+
31	-	-	+
32	-	-	+
33	-	+/-	+
34	-	+/-	+
35	-	+/-	+
36	-	-	+
37	-	-	+
38	-	-	+
39	-	-	+
40	-	-	+
41	-	-	+
42	-	-	+
43	-	-	+
44	++	++	+
45	-	-	+
46	-	-	+
47	-	-	+
48	-	-	+
49	-	-	+
50	-	-	+

Figure 9 X-gal Assay to test the expression of LacZ *reporter gene by the clones fished for EHS-1 125 (3rd EH domain) Y2H screening*

A,B,C,D,E: Yeast Control strains transformed with plasmids containing no inserts (A), or transformed with plasmids expressing interactors of increasing interaction strengths in the order of B-E, as described in detail in Table 1 in Section 6.2.3.

a. EHS-1 125 Y2H screening I: X-gal assays of a total of 50 clones that were identified as positive for the expression of *HIS3* with 5mM [3AT].

b. EHS-1 125 Y2H screening II: X-gal assays of a total of 25 clones that were identified as positive for the expression of *HIS3* with the determined [3AT].

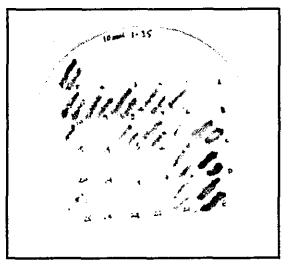


Table 8 The expression of the reporter genes, LacZ, *and* URA3, *by the EHS-1 interacting-clones that were initially selected for the expression of* HIS3 *in the presence of 10mM 3AT.*

The levels of expression were compared with that of the control strains A-E as follows: A:-, B: +, C: ++, D:+++, E: ++++. Note that the intensity shown in the figure may not correspond exactly to that observed during the experiment as the intensity of the colour changed during storage of the membrane after the experiment.

Clone #	*LacZ*	*URA3*	*HIS3*
1	-	-	+
2	-	++	+
3	-	++	+
4	-	-	+
5	++	-	+
6	+	-	+
7	+	-	+
8	++	-	+
9	++	-	+
10	++	-	+
11	+	-	+
12	+	-	+
13	+	-	+
14	-	-	+
15	+	-	+
16	+	-	+
17	-	+/-	+
18	-	+/-	+
19	-	-	+
20	-	-	+
21	+	-	+
22	-	-	+
23	-	-	+
24	-	-	+
25	+	-	+

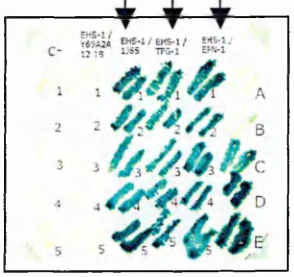
7.2.4 Interactors of EHS-1

A total of 31 putative interactors of EHS-1 were identified as cDNA inserts in-frame with GAL4AD. Following the re-transformation assays, 14 proteins were confirmed as putative interactors of EHS-1: EPN-1, TFG-1, D1081.7 (1J65), LIN-10, DAB-1, PQN-32, F15C11.2, Y45F10D.13, F46H5.7 (also referred to as XH858), B0041.2, C50C3.8, CAS-1, Y11D7A.12 and ALX-1 (Figure 10).

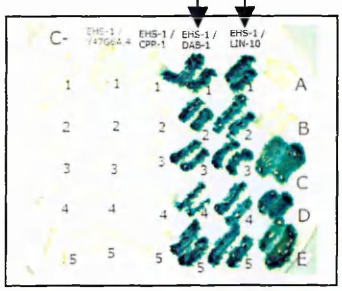
Figure 10. The X-gal Assays used as an assay for the Re-transformation assay of the putative positive interactors that were identified from the Y2H screenings with EHS-1.

A,B,C,D,E: Yeast Control strains transformed with plasmids containing no inserts (A), or transformed with plasmids expressing interactors of increasing interaction strengths in the order of B-E, as described in detail in Table 1 in Section 6.2.3.
1,2,3,4,5: Five different co-transformants tested in order to ensure that the LacZ-activation was not observed not only in one clone but in multiple clones.
The positive interactions detected in the X-gal assays are marked with arrows.

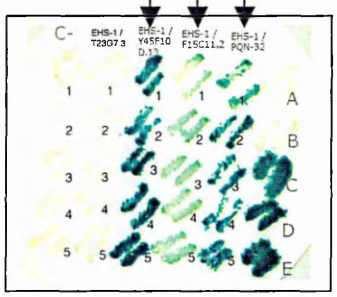
1) EPN-1, TFG-1, D1081.7 (1J65) and



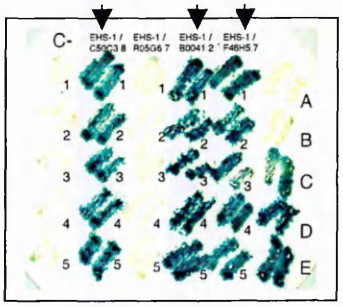
2) LIN-10, DAB-1, CPR-1, and Y47G6A.4



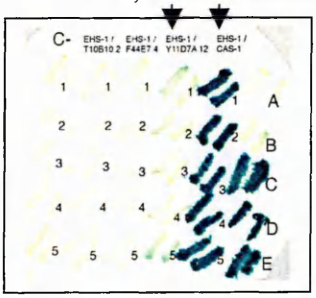
3) PQN-32, F15C11.2, Y45F10D.13 and T23G7.3



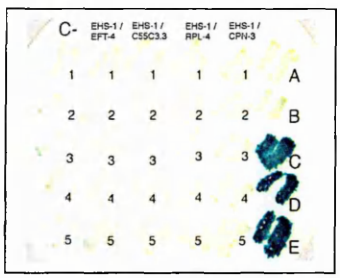
4) F46H5.7, B0041.2, R05G6.7 and C50C3.8



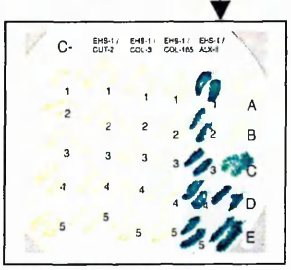
5) CAS-1, Y11D7A.12, F44E7.4, and T10B10.2



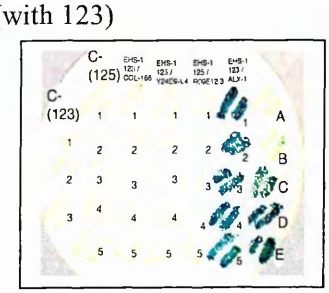
6) CPN-3, RPL-4, C55C3.3 and EFT-4



7) ALX-1, COL-165, COL-3, and CUT-2



8) 123/ALX-1 (positive control), R09E12.3 (with 125), Y24D9A.4 (with125), COL-166 (with 123)



7.3. RME-1 Y2H screenings

7.3.1 Preparation of RME-1 Bait D

The bait to be used for the Y2H screenings for RME-1 was initially cloned to contain the restricted region surrounding the EH domain at the C-terminus (Bait B, Figure 1c). However, this construct was not expressed and another bait, Bait D was constructed. Bait D contains an extension of 49 amino acids in the region N-terminal to the EH domains (Bait D, Figure 1c). Bait D was expressed in-frame with the GAL4DBD, as detected as the band at the expected molecular weight of 35.1kDa, recognized by an anti-GAL4DBD Western Blot (Figure 2a). Bait D was also confirmed not to self-activate *LacZ* gene, as shown in Figure 3d. The concentration of 3AT required to titrate out the basal *HIS3* expression in the co-transformants of bait D and the empty prey vector was determined to be 10mM (Figure 5c).

7.3.2 Y2H screening with RME-1Bait D

In spite of the fact that the total co-transformation efficiency for Bait D had reached more than the required level of 1 x 10^6 colonies, only 1 clone was selected for *HIS3* expression in the presence of 10mM 3AT. This clone was shown to express *LacZ* (Figure 11) but not *URA3* (result not shown). Two plasmids were isolated from this clone, and sequencing of each plasmid identified ASP-1, ALX-1, and GLY-8. Two rounds of re-transformation assays confirmed only ALX-1, although the expression of *LacZ* detected by the X-gal assay was very weak (Figure 12).

7.3.3 Preparation of other RME-1 baits

In an attempt to find a bait that might act as a functional EH domain of RME-1 without having to resort to using the full-length protein, 8 more constructs, containing different regions of RME-1, were prepared (see baits A, C, E, F, G, H, I, J in Figure 1c). In order to test whether the EH domains contained in these baits successfully interacted with a

putative interactor, they were co-transformed with cDNAs inserts that were identified to interact with the other EH domains of ITSN-1 and EHS-1 in the previously described screenings. This approach was chosen because it requires only small-scale transformations of yeast, rather than a library-scale screening.

Figure 11. X-gal Assay to test the expression of **LacZ** *reporter gene by the clone fished by RME-1 Y2H Screenings.*

The clone marked as RME-1 Y2H #1 is the only clone to be concerned in this figure. The other unlabelled clones are unrelated clones from the ITSN-1 Y2H screenings.

A,B,C,D,and E are the control strains co-transoformed with two Y2H plasmids with no inserts (A), ot with Y2H plasmids expressing interactors of increasing strengths from B to E. The details of each strain is described in Table 1 in the section 6.2.3.

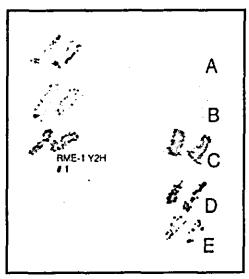
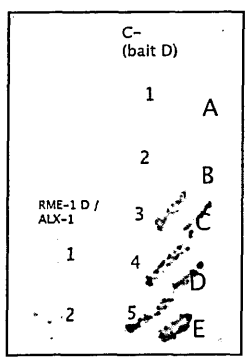


Figure 12. Re-transformation assay of the putative positive interactors that were identified from the RME-1 Y2H screening I using bait D.

The level of expression of LacZ by the re-transformed clones observed was very weak. The interaction was considered positive nonetheless, for the fact that the expression positive with respect to the control strain A (A), the control strain B which should be expressing a weak interaction, and the negative control of the bati D alone (D).

A,B,C,D,and E are the control strains co-transoformed with two Y2H plasmids with no inserts (A), ot with Y2H plasmids expressing interactors of increasing strengths from B to E. The details of each strain is described in Table 1 in the section 6.2.3.

1,2,3,4,5: Five different co-transformants tested in order to ensure that the self-activation was not observed not only in one clone but in multiple clones.



The cDNAs of interactors that were used to test the various RME-1 baits were: epn-1, F15C11.2 and alx-1. The first two were chosen as they had been frequently identified from the cDNA library by both ITSN-1 and EHS-1. The cDNA encoding ALX-1 was used as it was the only interactor identified from the Y2H screening with the RME-1 bait D. Furthermore, all of them contained at least one NPF motif, which is a known interacting motif of EH domains [11].

Each bait was co-transformed with the empty prey vector or with the EH-interacting cDNAs. The co-transformants were tested for the activation of *LacZ* reporter gene by X-gal assay (Figure 4). The co-transformations were carried out also with EHS-1 Bait 123 as a positive control. The expression of each bait in-frame with GAL4DBD was checked by anti-GLA4DBD Western Blot. RME-1 Bait D, F and H showed successful binding to ALX-1.

Bait J also interacted with ALX-1, but was considered unsuitable for the screenings, because of the presence of the coiled-coil region, which could identify interactors that are not specific for the EH domain. Nonetheless, in view of the efficiency in interacting with NPF containing proteins, Bait J was used in the re-transformation assays with the putative clones identified from a screening with EH domain only. Bait J was preferred to other baits for the purpose of confirmation, in the hope that the extended region N-terminal to the EH domain may facilitate the correct folding of the bait.

The expression of Baits F, H and J in-frame with GAL4DBD detected by anti-GAL4DBD Western blot is shown in Figure 2b. The concentration of 3AT required to titrate out the *HIS3* gene for bait F was also found to be much higher than that of the other baits, between 60mM and 75mM. In comparison, the concentration of 3AT

required for the selection of *HIS3* expression during the screening was determined to be in the range of 30mM to 40mM. Unfortunately, Bait F showed self-activation of *LacZ* gene whereas Bait H did not.

The fact that Bait F represented the minimal region that interacted with all the NPF-containing cDNAs made Bait F still more favourable to Bait H. Following the progress of the X-gal assay closely every hour suggested that the self-activation of *LacZ* would take place after 5-6 hours of incubation at 37°C, approximately when the control strain C become positive. Using this information, an attempt at an Y2H screening was made.

7.3.4 Y2H screenings with RME-1 bait F (I)

A total of 175 clones were picked for the expression of *HIS3* in the presence of 60mM 3AT. 20 out of these expressed *LacZ* earlier than the bait alone (C-) started to self-activate the expression of *LacZ*. The positive clones were marked with red spots on the membrane in order to distinguish them from the others that turned blue due to the self-activation by the bait (results not shown). Unfortunately no clones were selected for *URA3* expression.

7.3.5 Y2H screening with RME-1 bait F (II)

In the presence of 75mM of 3AT, 50 clones were selected *HIS3* expression under 75mM 3AT. The X-gal assay of these clones showed that only 1 clone, #22 expressed *LacZ*, but very slightly. No clone expressed *URA3* (results not shown) The sequencing clone #22 identified ncam-1, but was out-of-frame with respect to GAL4AD. The cDNA was checked by re-transformation, but did not show interaction with RME-1 bait J.

7.3.6 Y2H screening with RME-1 bait H (I)

Co-transformation of the bait construct H with the cDNA library identified 75 clones for *HIS3* expression in the presence of 30mM of 3AT. However, no clone expressed *LacZ* expression that could be detected by X-gal assays (results not shown), and no clones showed the expression of *URA3* more than that shown by the bait alone (C-) (results not shown).

7.3.7 Y2H screening with RME-1 Bait H (II)

A more stringent round of screening with Bait H was selected for HIS3 expression in the presence of 40mM 3AT. A total of 40 clones were selected for *HIS3* expression. 1 clone out of 40 showed a weak expression of *LacZ* (results not shown), but no clone showed the expression of *URA3* (results not shown). Sequencing of the clone #32 showed that the cDNA insert encoded CYN-6. However, CYN-6 was not confirmed in the re-transformation assays.

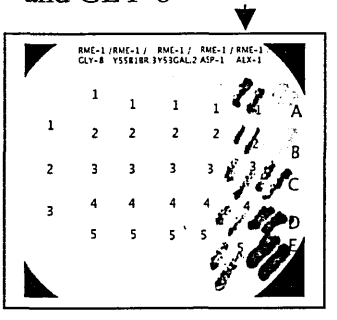
7.3.8 Interactor of RME-1

21 putative interactors that were identified from a total of five Y2H screenings with three different bait constructs of RME-1, Bait D, F, and H were re-transformed with Bait J. The only protein, which was identified and confirmed, was ALX-1. The co-transformant of ALX-1 and bait J was also used as the positive control for each membrane of X-gal assays (Figure 13).

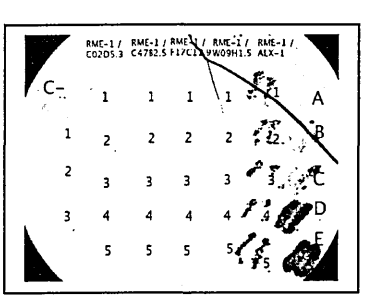
Figure 13The X-gal Assay used as an assay for the re-transformation assay of the putative positive interactors that were identified from the RME-1 Y2H screenings I-VI with bait J.

The re-transformation with ALX-1 with Bait J was used as a positive control (One example is indicated with an arrow). ALX-1 was used as it was already shown to be a positive interactor with shorter bait, Bait D in the previous section (Figure 12).
A,B,C,D,E: Yeast Control strains transformed with plasmids containing no inserts (A), or transformed with plasmids expressing interactors of increasing interaction strengths in the order of B-E, as described in detail in Table 1 in Section 6.2.3.
1,2,3,4,5: Five different co-transformants tested in order to ensure that the LacZ activation was not observed not only in one clone but in multiple clones.

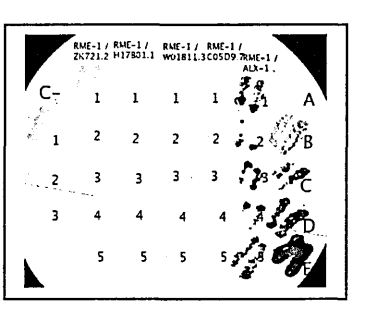
1) ALX-1, ASP-1, Y53GAL.2, Y55B1BR.3, and GLY-8



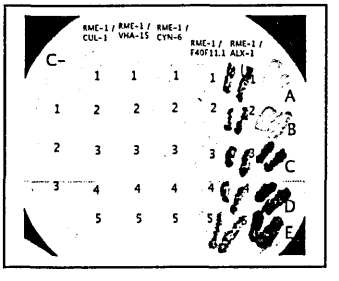
2) ALX-1, W09H1.5, F17C11.9, C47B2.5, and C02D5.3



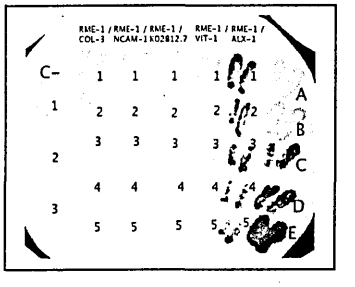
3) ALX-1, C05D9.7, W01B11.3, H17B01.1, and ZK721.2



4) ALX-1, F40F11.1, CYN-6, VHA-15, and CUL-1



5) ALX-1, VIT-1, K02B12.7, NCAM-1, and COL-3



7.4. REPS-1 Y2H screenings

7.4.1 Preparation of REPS-1 baits

Three baits were constructed in total for the EH domain of REPS-1. The 3AT assay for Bait A indicated that 20mM of 3AT was sufficient to titrate the background expression of *HIS3* gene. However, once the bait was co-transformed with the cDNA library in two attempted screenings, a large number, over a thousand colonies per plate (10 cm diam.) were found even in the presence of 40mM, suggesting the background expression of *HIS3*. Bait B, which was designed to contain a limited but a different region around the EH domain, starting at 30bp downstream closer to the EH domain but contains 134bp more after the domain in comparison to Bait A (Figure 1d. REPS-1 Bait B). Unfortunately, an attempt at a screening with Bait B exhibited the same problem as Bait A.

7.4.2 REPS-1 Rapid Amplification of the cDNA Ends (RACE)

Despite the immense information available on thousands of genes in *C. elegans*, REPS-1 gene was poorly characterised at the time of screening. No expression pattern was known, and there was no EST available that corresponded to the full-length sequence of REPS-1. The sequences that were publicly available on the database at http://www.wormbase.org, were ambiguous, as they were modified a several times even though the exon encoding the EH domain remained constant. Therefore, in order to determine the 5' and the 3'end of REPS-1 sequence, 5'RACE and 3' RACE was carried out to determine the sequence of REPS-1 present in the genomic DNA extracted from *C. elegans*.

Combining both of the amplified sequences of the 5' end and the 3' end suggested two isoforms of REPS-1. The amino acid sequences of two isoforms are shown in Figure 14, alongside the amino acid sequence that is currently available on the Wormbase.

Figure 14 The amino acid sequence found in the Wormbase and two possible amino acid sequences of Reps-1 that was determined by the combination of the translated sequences identified by 5' RACE and 3' RACE.

(The region identified as the EH domain by the NCBI Conserved Domain search at:

http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi is shown in red)

1. WORMBASE SEQUENCE:
>WP:CE37856
MTENDKKSQYFSNGDFEKVAADIISSPRRLKHTSPADSNADSLFKITEKQ
QEYYTKCFRHLIKTTQGAADLCGALCGADQRIVAFFKRSSLDMSSLSKIW
SLADVNEDGWLDLNEFSIAMHLVVLKVKGEVPIPDVLPGFARPPLTEPRA
PSTVAATPSPAGAGHVEADGPVKNWANQPIIKQFSDTPPLLVDSRPTAIK
HSALLALKSPLGPPPIPPVRPQQQQRGHNRSASLDLKLIALNKTKASAES
QLPPTTLSLWSSHSDPTAQSISTTTTTTTFASFPATPDSIPPPIPQRITP
SPLPRVIEEEKRITTTDSSTQTTELLYSEEDVKSFFSKIGHQIDDLIGEE
IQATDGQGIERWKERCTALRTQNSELEAERARLAQVRIQLEIRIQEFEER
SKTACSSSTL

2. LONG ISOFORM:
371aa
MTENDKKSQYFSNGDFEKVAADIISSPRRLKHTSPADSNADSLFKITEKQQ
EYYTKCFRHLIKTTQGAADLCGALCGADQRIVAFFKRSSLDMSSLSKIWSL
ADVNEDGWLDLNEFSIAMHLVVLKVKGEVPIPDVLPGFTRPPLTEPRAPST
VAATPSPAGAGHVEADGPVKNWANQPIIKQFSDTPPLLVDSTPTAIKHSAL
LALKSPLGPPPIPPVRPQQQQRGHNRSASLDLKLIALNKTKASAESQLPPT
TLSLWSSHSDPTAQSISTTTTTTTFASFPATPDSIPPPIPQRITPSPLPRV
IEKKRTYSESY

3. SHORT ISOFORM:
181aa
MTENDKKSQYFSNGDFEKVAADIISSPRRLKHTSPADSNADSLFKITEKQQ
EYYTKCFRHLIKTTQGAADLCGALCGADQRIVAFFKRSSLDMSSLSKIWSL
ADVNEDGWLDLNEFSIAMHLVVLKVKGEVPIPDVLPGFTRPPLTEPRAPST
VAATPSPAGAGHVEADGPVKNVGVFEII

7.4.3. Preparation of REPS-1 Bait C

Bait C, a larger construct which contains a region surrounding the EH domain, was amplified by PCR using the mixture of 5'RACE reaction and the 3' RACE reaction as the template. The expression of Bait C in-frame with the GAL4DBD was demonstrated by the anti-GAL4DBD Western Blot (Figure 2). The concentration of 3AT required to titrate out the background expression of *HIS3* for Bait C was determined to be 10mM, and the bait was used in a total of two screenings under the presence of 10mM 3AT.

7.4.4 Y2H screenings with REPS-1 Bait C

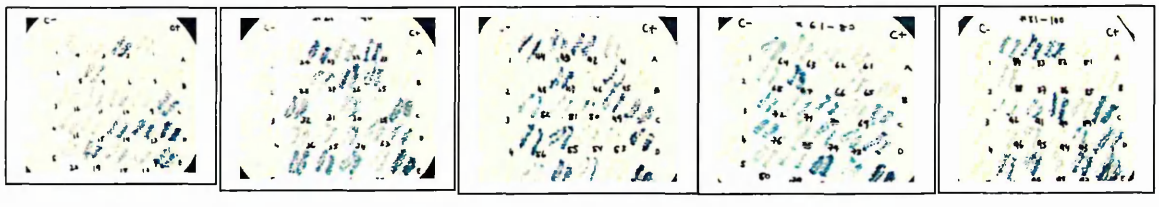
A total of 140 clones were selected for *HIS3* expression in the presence of 10mM 3AT. 77 out of these clones were positive for *LacZ* reporter gene expression (Figure 15a), whereas 44 clones expressed *URA3* (results not shown). The expression of three reporter genes by the clones isolated in this screening is summarised in Table 9.

A less stringent screening in the presence of 5mM of 3AT resulted in the selection of 120 clones for *HIS3* expression. X-gal assays of the clones identified 38 positive clones (Figure 15b), whereas the selective medium lacking Uracil identified 26 clones (results not shown). The expression of three reporter genes by the clones isolated in this screening is shown in Table 10.

Figure 15X-gal Assay to test the expression of* LacZ *reporter gene by the clones fished by Reps-1 Y2H screenings.

A,B,C,D,E: Yeast Control strains transformed with plasmids containing no inserts (A), or transformed with plasmids expressing interactors of increasing interaction strengths in the order of B-E, as described in detail in Table 1 in Section 6.2.3.

a. Reps-1 Y2H screening I: X-gal assays of a total of 140 clones that were identified as positive for the expression of *HIS3* with 10mM [3AT].



b. Reps-1 Y2H screening II: X-gal assays of a total of 120 clones that were identified as positive for the expression of *HIS3* with 5mM [3AT].

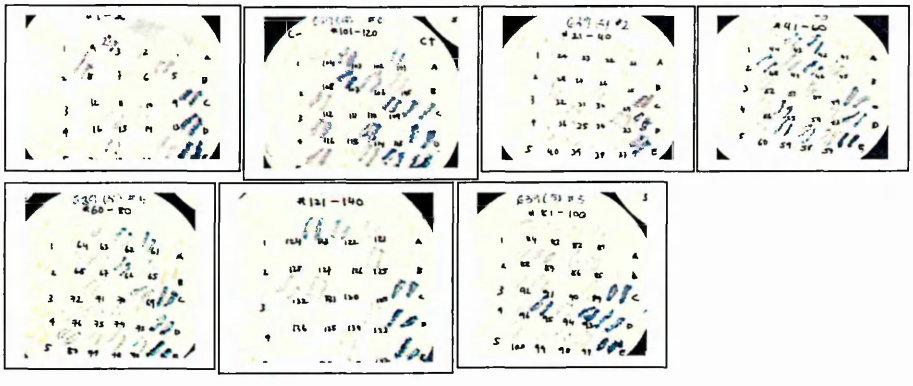


Table 9 The expression of the reporter genes, **LacZ,** *and* **URA3,** *by the REPS-1 interacting-clones that were initially selected for the expression of* **HIS3** *in the presence of 10mM 3AT.*

The levels of expression were compared with that of the control strains A-E as follows: A:-, B: +, C: ++, D:+++, E: ++++. Note that the intensity shown in the figure may not correspond exactly to that observed during the experiment as the intensity of the colour changed during storage of the membrane after the experiment.

Clone #	*LacZ*	*URA3*	*HIS3*
1	+	-	++
2	+++	+++	++
3	+	-	++
4	+	-	++
5	-	-	++
6	-	-	++
7	++	++	++
8	-	-	++
9	-	+/-	++
10	+	-	++
11	+	-	++
12	+	-	++
13	+++	++	++
14	+++	-	++
15	+	-	++
16	++	-	++
17	+/-	-	++
18	+	-	++
19	+++	+++	++
20	+	-	++
21	++++	++++	++
22	+	-	++
23	++++	++++	++
24	-	-	++
25	+++	-	++
26	++++	+++	++
27	++	+	++
28	-	-	++
29	-	-	++
30	+++	+	++
31	-	-	++
32	+++	+	+
33	+	-	+
34	++	++	+
35	-	-	+
36	-	-	+
37	-	-	+
38	++	-	+
39	+++	-	+
40	+++	+++++	+
41	-	+/-	+
42	++	+/-	+
43	++	+	+
44	+++	+++	+
45	++	++	+
46	-	-	+
47	+++	++	+
48	-	-	+
49	+++	++	+
50	+	-	+
51	-	-	+
52	+	++	+
53	-	-	+
54	-	-	+
55	+++	+	+
56	++++	+++	+
57	-	-	+
58	++	-	+
59	+++	+	+
60	+	-	+
61	-	-	+
62	-	-	+
63	-	-	+
64	+/-	-	+
65	+/-	-	+
66	-	-	+
67	+++	+++	+
68	-	-	+
69	-	-	+
70	++	+++	+
71	+/-	-	+
72	++	+	+
73	+	-	+
74	++	-	+
75	+	-	+
76	+	+	+
77	+	-	+
78	+++	+	+
79	-	+/-	+
80	-	+	+
81	-	-	+
82	+++	++	+
83	++++	+++	+
84	++	++	+
85	-	-	+
86	-	-	+
87	-	-	+
88	+/-	-	+
89	+/-	-	+
90	++++	+++	+
91	+	-	+
92	+	-	+
93	+	-	+
94	-	-	+
95	+	-	+
96	-	-	+
97	+++	++	+
98	-	-	+
99	+++	++	+
100	+++	+++	+
101	++	+	+
102	-	-	+
103	++	+++	+
104	-	+/-	+
105	-	-	+
106	++	+/-	+
107	+++	+++	+
108	-	-	+
109	+++	++	+
110	-	-	+
111	-	-	+
112	+	-	+
113	-	-	+
114	+++	+++	+
115	-	-	+
116	++	-	+
117	+++	+	+
118	++	-	+
119	-	-	+
120	++	+++	+
121	-	-	+
122	+	-	+
123	++	+++	+
124	-	+	+
125	+	+/-	+
126	-	-	+
127	-	-	++
128	-	-	+
129	-	-	+
130	-	-	+
131	+	++	+
132	+	-	+
133	+/-	-	+
134	-	+/-	+
135	-	-	+
136	-	-	+
137	-	-	+
138	+/-	+/-	+
139	-	+/-	+
140	-	-	+

Table 10 The expression of the reporter genes, **LacZ** *and* **URA3**, *by the REPS-1 interacting-clones that were initially selected for the expression of* **HIS3** *in the presence of 5mM 3AT.*

The levels of expression were compared with that of the control strains A-E as follows: A:-, B: +, C: ++, D:+++, E: ++++. Note that the intensity shown in the figure may not correspond exactly to that observed during the experiment as the intensity of the colour changed during storage of the membrane after the experiment.

Clone #	*LacZ*	*URA3*	*HIS3*
1	-	+	++
2	-	+	++
3	+++	++++	++
4	-	-	++
5	+	-	++
6	-	-	++
7	-	-	++
8	++	+++	++
9	-	-	++
10	-	-	++
11	-	-	++
12	-	-	++
13	-	-	++
14	+	-	++
15	++	-	++
16	-	++	++
17	+/-	-	++
18	-	-	++
19	+	-	++
20	-	-	++
21	+/-	+	++
22	-	-	++
23	-	+	++
24	+/-	-	++
25	-	-	++
26	-	-	+/-
27	-	-	++
28	-	-	++
29	-	-	++
30	+	+/-	++
31	-	-	++
32	+/-	-	++
33	+	-	++
34	-	-	++
35	+	++	++
36	-	-	++
37	-	-	++
38	-	-	++
39	-	-	++
40	-	++	++
41	-	+/-	++
42	+++	++	++
43	+	-	++
44	-	-	++
45	+	-	++
46	+++	+++	++
47	+	-	++
48	++	-	++
49	-	-	++
50	+	-	++
51	-	-	++
52	+/-	-	++
53	+/-	-	++
54	-	-	++
55	+++	+++	++
56	-	-	++
57	+/-	-	++
58	++	-	++
59	+++	+++	++
60	-	-	++
61	++	-	++
62	+	-	++
63	-	+	++
64	-	-	++
65	+/-	-	++
66	++	++	++
67	-	+	++
68	-	-	++
69	+	+	++
70	-	+/-	++
71	-	-	++
72	-	-	++
73	-	-	++
74	-	-	+
75	-	-	+
76	-	-	+
77	+	-	+
78	-	+	+
79	-	-	+
80	+	-	+
81	-	-	+
82	-	-	+
83	-	-	+
84	+/-	-	+
85	-	-	+
86	+	+	+
87	+	+	+
88	+	-	+
89	-	-	+
90	-	-	+
91	+/-	-	+
92	+/-	-	+
93	+++	+++	+
94	+	-	+
95	+++	+++	+
96	-	-	+
97	-	-	+
98	+	-	+
99	-	-	+
100	+	-	+
101	-	-	+
102	-	-	+
103	-	-	+
104	-	-	+
105	-	-	+
106	++	+	+
107	+++	+	+
108	-	-	+
109	-	-	+
110	-	-	+
111	-	-	+
112	-	-	+
113	+	++++	+
114	-	-	+
115	-	++++	+
116	+++	-	+
117	-	-	+
118	+/-	-	+
119	+	-	+
120	+/-	-	+

7.4.5 Interactors of REPS-1

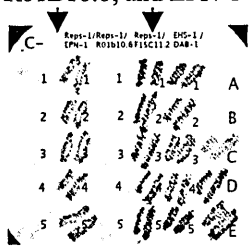
A total of 63 distinct interactors were identified from the two Y2H screenings with REPS-1 EH domain. Following the re-transformation, a total of 14 proteins were confirmed as putative interactors of REPS-1, which are: F15C11.2, EPN-1, SEL-5, T05F1.4, PCN-1, VAB-19, ALH-9, LIN-10, DAB-1, B0041.2, T23G11.7 (1I257), M03A8.3 (XH423), F23B12.5 (5O926) and Y37E3.11 (1C653), as shown in Figure 16.

Figure 16. The X-gal Assays used as an assay for the Re-transformation assay of the putative positive interactors that were identified from the Reps-1 Y2H screening.

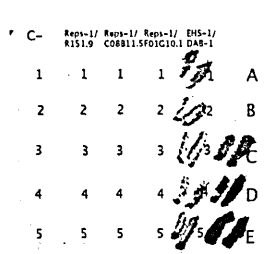
The co-transformants of EHS-1 with DAB-1 cDNA were used as positive controls on each membrane, whereas co-transformants of the Reps-1 bait B was used as the negative controls.
A,B,C,D,E: Yeast Control strains transformed with plasmids containing no inserts (A), or transformed with plasmids expressing interactors of increasing interaction strengths in the order of B-E, as described in detail in Table 1 in Section 6.2.3.
1,2,3,4,5: Five different co-transformants tested in order to ensure that the LacZ activation was not observed not only in one clone but in multiple clones.

The positive interactions detected in these X-gal assays are indicated with arrows.

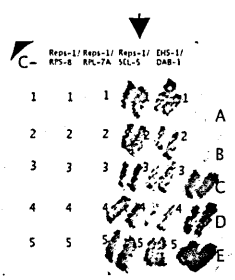
1) EHS-1/DAB-1 (Positive control), F15C11.2, R01B10.6, and EPN-1



2) Positive control, F01G10.1, C08B11.5, and R151.9

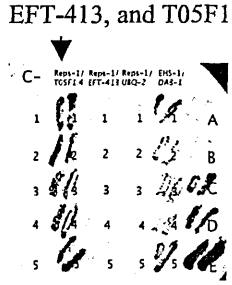


3) Positive control, SEL-5, RPL-7A, and RPS-8

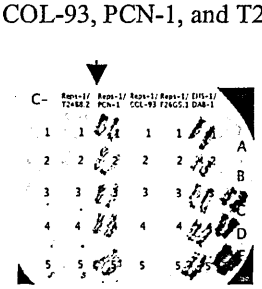


4) Positive control, CPL-1, RPS-7, and HIM-3

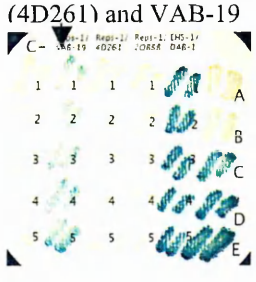
5) Positive control, UBQ-2, EFT-413, and T05F1.4



6) Positive control, F26G5.1, COL-93, PCN-1, and T24B8.2

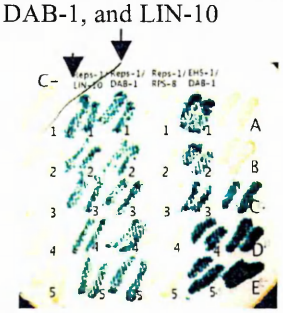


7) Positive control, F19H8.2 (2O888), Y69A2A12.18 (4D261) and VAB-19

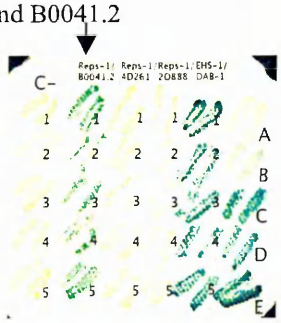


8) Positive control, ALH-9, RPL-15, and COL-3

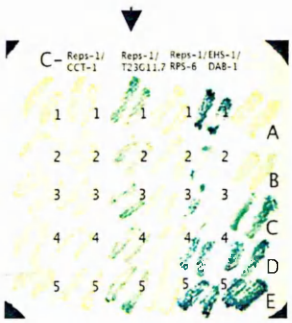
9) Positive control, RPS-8, DAB-1, and LIN-10



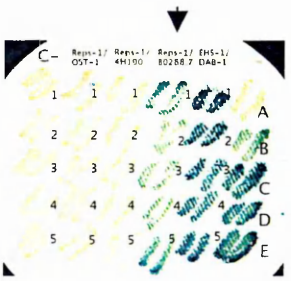
10) Positive control, F19H8.2 (2O888), Y69A2A5.18 (4D261) and B0041.2



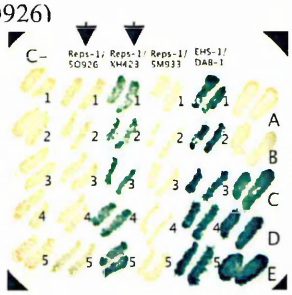
11) Positive control, RPS-6, T23G11.6, and CCT-1



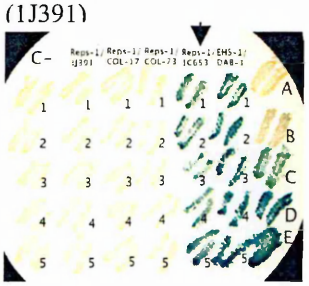
12) Positive control, B0288.7, Y37B6BL.24 (4H190), and OST-1



13) Positive control, F28C1.1, M03A8.3 (XH423), and F23B12.5 (5O926)



14) Positive control, Y37E3.11, COL-73, COL-17, and F26A2.7 (1J391)



7.5. R10E11.6 Y2H Screenings

Although γ-synergin was classically thought to be a mammalian specific protein, when the gene R10E11.6 was reported to encode for an EH domain, the BLAST search of its amino acid sequence identified other sequences homologous to γ-synergin in various species. The species ranged from a sea urchin species, Strongylocentroutus purpuratus, bird species (Gallus gallus), and primates (Macaca mulatta, and Pan troglodytes) to human.

7.5.1 Preparation of R10E11.6 bait

The EH domain of R10E11.6 was estimated in the region between 75aa to 120aa, corresponding to 225bp-360bp by the conserved domain search engine available on the website of the NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Even though the region of 45 amino acids detected as EH domain was much smaller than what is usually expected of other EH domains, which is approximately of 100 amino acids, the region restricted to the area from 225bp to 462bp was cloned as the first bait (Bait A in Figure 1e). However, the expression of the bait in-frame with the GAL4DBD could not be detected successfully by anti-GAL4DBD Western Blot. When the conserved domain search was repeated, the alignment with the other known EH domains identified EH domain region started from 58aa up to 118aa. Therefore, another bait (Bait B) was cloned containing the region from 166bp till 770bp corresponding to the larger area recognized as an EH domain. The expression in-frame with GAL4DBD was detected successfully and the X-gal assay of the co-transformants with an empty prey vector showed that the bait did not self-activate the expression of *LacZ* (Figure 3e). The minimum concentration of 3AT required to titrate out the basal *HIS3* expression of the R10E11.6 bait was determined to be 10mM (Figure 3d).

7.5.2 Y2H screenings with R10E11.6 Bait B

From the first round of co-transformation, 25 clones were selected for *HIS3* expression in the presence of 10mM 3AT. Only the clone #1 was found to be positive for *LacZ* expression (Figure 17), and no clone expressed *URA3* (results not shown). From two additional rounds of screenings, a total of 100 clones were selected for HIS3 expression. Only one clone, #57 from the second screening was shown to express *URA3*, but not *LacZ* (Figure 17b). As a result, three screenings identified two clones.

The clone #1 from the first screening was identified to encode for ZK822.5. Two plasmids were isolated from the clone #57, and both were sequenced. One plasmid was found to encode for pdi-3, whereas the other plasmid encoded for a peptide sequence unrecognizable as any specific *C. elegans* protein. All three cDNAs were re-transformed with the EH domain of R10E11.6 but none of them showed positive *LacZ* expression (Figure 18).

7.5.3 The EH domain of R10E11.6

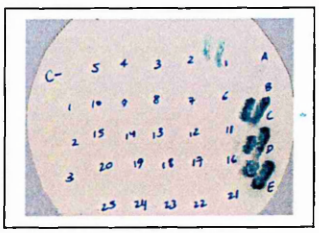
Y2H screening during this project identified no putative interactors of R10E11.6. As mentioned previously in Section 2.5, γ-synergin binds to SCAM-1 through an EH-NPF interaction. In order to check whether the EH domain of R10E11.6 is able to perform an interaction similar to that shown by the EH domain of the mammalian γ-synergin, the *C. elegans* homologue of SCAMP1 (Secretory Crier Membrane Protein 1), SCM-1, was cloned in-frame with the GAL4AD. No interaction was found between the R10E11.6 EH domain and the SCM-1 using the Y2H approach (Figure 19). The fact that the EH domain of R10E11.6 was not shown to interact with any protein, including the predicted interactor SCM-1, raises the question of whether the R10E11.6 gene product contains a *bona fide* EH domain-containing protein, and requires further investigation.

Figure 17. X-gal Assay to test the expression of LacZ *reporter gene by the clones fished in R10E11.6 Y2H Screenings.*

A,B,C,D,E: Yeast Control strains transformed with plasmids containing no inserts (A), or transformed with plasmids expressing interactors of increasing interaction strengths in the order of B-E, as described in detail in Table 1 in Section 6.2.3.

Three yeast clones co-transformed with the bait construct pDBLeu-R10E11.6 and the empty prey vector pPC86 were used as the negative control, as well as the control strain A.

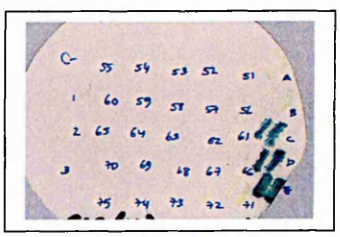
a. R10E11.6 Y2H screening I: A total of 25 clones that were identified as positive for the expression of *HIS3* with 10mM [3AT].



b. R10E11.6 Y2H screening II: A total of 75 clones that were identified as positive for the expression of *HIS3* with 10mM [3AT].

This figure shows Clone #57, the only clone identified in this screening.

Clone #57 expressed of *URA3* but not *LacZ*.



c. R10E11.6 Y2H screening III: A total of 25 clones that were identified as positive for the expression of *HIS3* with 10mM [3AT]

Figure 18. The X-gal Assays used as an assay for the Re-transformation assay of the putative positive interactors that were identified from the R10E11.6 Y2H screening I with bait B under the presence of 10mM 3AT for the initial **HIS3** *selection.*

A,B,C,D,E: Yeast Control strains transformed with plasmids containing no inserts (A), or transformed with plasmids expressing interactors of increasing interaction strengths in the order of B-E, as described in detail in Table 1 in Section 6.2.3.

Five yeast clones co-transformed with the bait construct pDBLeu-R10E11.6 and the empty prey vector pPC86 were used as the negative control, as well as the control strain A.

1,2,3,4,5: Five different co-transformants tested in order to ensure that the *LacZ* activation was not observed not only in one clone but in multiple clones.

1) ZK822.4, (II)57-1, and PDI-3

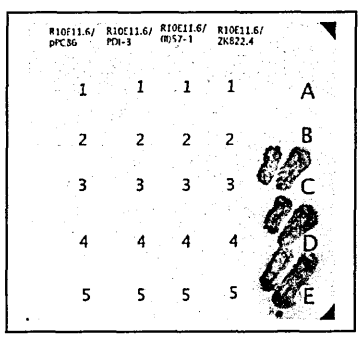
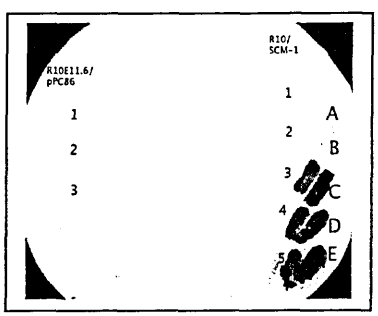


Figure 19. The X-gal Assay of the co-transformants of the bait R10E11.6 Bait B and GAL4AD-SCM1.

A,B,C,D,E: Yeast Control strains transformed with plasmids containing no inserts (A), or transformed with plasmids expressing interactors of increasing interaction strengths in the order of B-E, as described in detail in Table 1 in Section 6.2.3.

Three yeast clones co-transformed with the bait construct pDBLeu-R10E11.6 and the empty prey vector pPC86 were used as the negative control, as well as the control strain A.

1,2,3,4,5: Five different co-transformants tested in order to ensure that the LacZ activation was not observed not only in one clone but in multiple clones.



8. "Criss-Cross" Transformation

A total of 26 proteins were identified in Y2H screenings with the EH domains of four EH proteins in *C. elegans*: ITSN-1, EHS-1, RME-1, and REPS-1 (indicated by the + in white boxes in Table 10). The list of all the interactors of the four EH proteins in *C. elegans* indicated that there were several proteins that were common between two or in some cases three EH protein. In order to better understand the specificity of the interaction of each EH domain-containing proteins, all the putative interactors were tested for possible interactions with all five of the EH-domain containing proteins. This approach will herein be referred to as "criss-cross transformation".

Yeast strain *MaV203* was co-transformed with each EH bait construct and different prey constructs containing cDNA inserts encoding the putative interactors identified in the Y2H screening of the other three EH proteins. The co-transoformants were tested heir expression of the reporter gene, *LacZ,* by X-gal assays. When there were several clones encoding the same gene, for example, *epn-1*, *dab-1* and *F15C11.2*, the shortest encoding cDNA inserts that were identified during the screenings were used. *LacZ* was chosen rather than the other reporter genes as it was shown to be more sensitive than *URA3* by many examples of clones during the Y2H screenings. In addition, *LacZ* showed more specificity in comparison to *HIS3* expression, considering the fact that many clones, which were positive for *HIS3* expression at the initial selection of clones for the Y2H screening were found to be negative for *LacZ* or *URA3* expression. Interactions were considered to be positive only when the results of the X-gal assays were confirmed from two independent transformations. Only one example of X-gal assay is shown for each putative interactor. The results of X-gal assays for the criss-cross transformations are shown separately for each EH protein: ITSN-1 (Figure 20), EHS-1 (Figure 21), RME-1 (Figure 22), REPS-1 (Figure 23) and R10E11.6 (Figure 24).

Figure 20. ITSN-1 Criss-Cross Transformation:

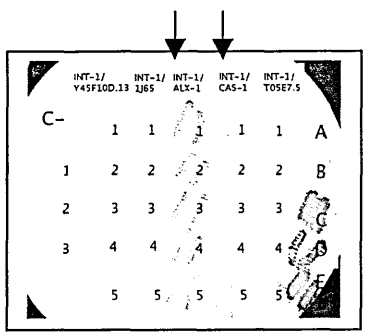
The X-gal Assays of clones that were transformed with ITSN-1 bait A and the cDNAs of the putative interactors that were identified from the Y2H screenings of EHS-1, RME-1, and Reps-1.
A,B,C,D,E: Yeast Control strains transformed with plasmids containing no inserts (A), or transformed with plasmids expressing interactors of increasing interaction strengths in the order of B-E, as described in detail in Table 1 in Section 6.2.3.

Three yeast clones co-transformed with the bait construct pDBLeu-ITSN-1 and the empty prey vector pPC86 were used as the negative control, as well as the control strain A.

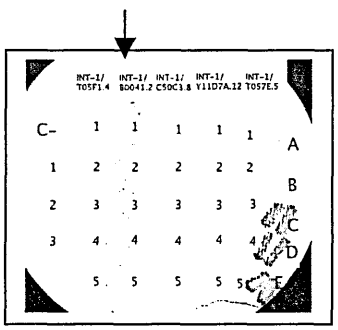
1,2,3,4,5: Five different co-transformants tested in order to ensure that the *LacZ* activation was not observed not only in one clone but in multiple clones.

Five co-transformants of the ITSN-1 bait and the prey expressing T05E7.5, a putative interactor of ITSN-1 previously identified in the Y2H screenings, were used as the positive control for each membrane.
The positive interactions are indicated with arrows.

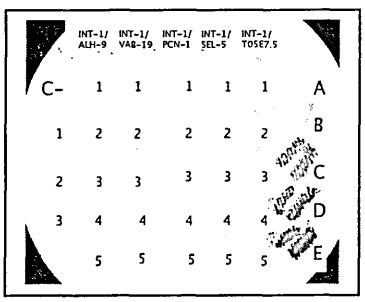
1) T05E7.5, CAS-1, ALX-1, D1081.7 (1J65), and Y45F10D.13



2) T05E7.5, Y11D7A.12, C50C3.8, B0041.2, and T05F1.4



3) T05E7.5, SEL-5, PCN-1, VAB-19, and ALH-9



4) T05E7.5, Y37E11.3 (1C653), M03A8.3 (XH423), F23B12.5 (5O926), and T23G11.7 (T25195)

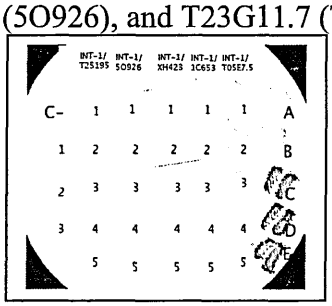


Figure 21 EHS-1 Criss-Cross Transformation:

The X-gal Assays of clones that were transformed with EHS-1 bait 123 and the cDNAs of the putative interactors that were identified from the Y2H screenings of ITSN-1, RME-1, and REPS-1.

A,B,C,D,E: Yeast Control strains transformed with plasmids containing no inserts (A), or transformed with plasmids expressing interactors of increasing interaction strengths in the order of B-E, as described in detail in Table 1 in Section 6.2.3.

Three yeast clones co-transformed with the bait construct pDBLeu-EHS-1 123 and the empty prey vector pPC86 were used as the negative control, as well as the control strain A.

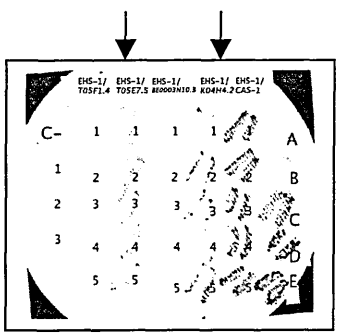
1,2,3,4,5: Five different co-transformants tested in order to ensure that the *LacZ* activation was not observed not only in one clone but in multiple clones.

Five co-transformants of the EHS1-1 bait and the prey expressing CAS-1, a putative interactor of ITSN-1 previously identified and confirmed in the Y2H screenings, were used as the positive control for each membrane.

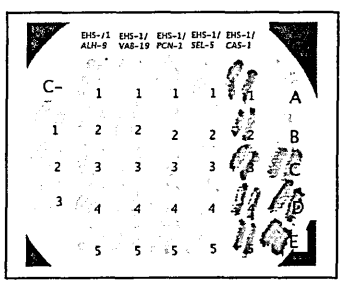
The scanned images of the membranes 2) and 3) appear as if there was a strong background activation of *LacZ* gene, as the negative controls are also coloured. However, the colour of the negative control clones during a total of 24 hours for the X-gal assay was equivalent to that shown by the negative control clones in the membrane 1). The difference in the colour of the clones in the membranes 2) and 3) is thought to be due to the fact that the membranes were not dried well when they were stored prior to the scanning of the images.

The positive interactions are indicated with arrows.

1) CAS-1, K04H4.2, BE0003N10.3, T05E7.5, and T05F1.4



2) CAS-1. SEL-5, PCN-1, VAB-19, and ALH-9



3) CAS-1, Y37E3.11 (1C653), M03A8.3 (XH423), F23B12.5 (5O926), and T23G11.7

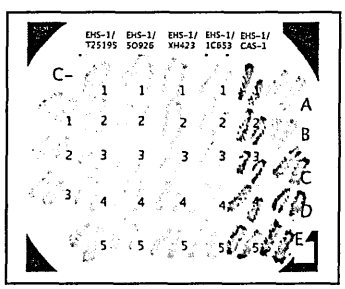


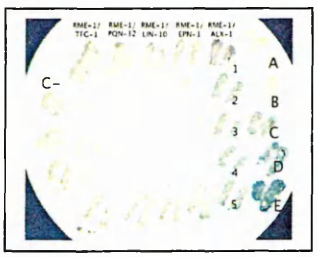
Figure 22. RME-1 Criss-Cross Transformation:

The X-gal Assays of clones that were transformed with RME-1 bait D and the cDNAs of the putative interactors that were identified from the Y2H screenings of ITSN-1, EHS-1, and REPS-1.
A,B,C,D,E: Yeast Control strains transformed with plasmids containing no inserts (A), or transformed with plasmids expressing interactors of increasing interaction strengths in the order of B-E, as described in detail in Table 1 in Section 6.2.3.
Three yeast clones co-transformed with the bait construct pDBLeu-RME-1 and the empty prey vector pPC86 were used as the negative control, as well as the control strain A.
1,2,3,4,5: Five different co-transformants tested in order to ensure that the *LacZ* activation was not observed not only in one clone but in multiple clones.
Five co-transformants of the RME-1 bait and the prey expressing ALX-1, a putative interactor of RME-1 previously identified and confirmed in the Y2H screenings, were used as the positive control for each membrane.

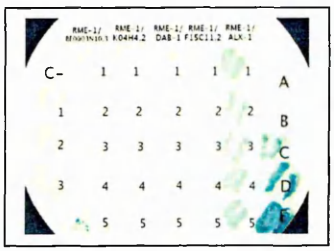
The co-transformants of the RME-1 bait and the prey fused with ALH-9 showed the activation of *LacZ* gene once (as shown in membrane 7)), but when the X-gal assay was repeated on clones from two different co-transformations, the activation of *LacZ* was not observed. In comparison, the *LacZ* activation was confirmed the interactions of RME-1 with Y37E3.11 and with Y11D7A.12.

The positive interactions are indicated with arrows.

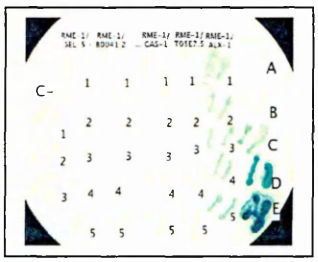
1) EPN-1, LIN-10, PQN-32, and TFG-1



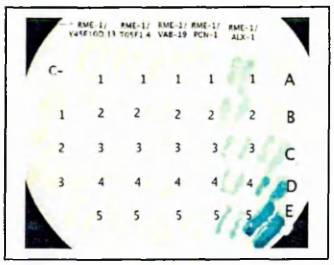
2) F15C11.2, DAB-1, K04H4.2, and BE0003N10.3



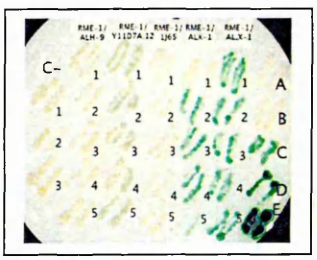
3) T05E7.5, CAS-1, B0041.2, and SEL-5



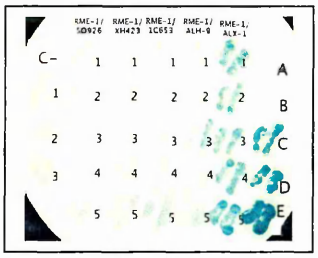
4) PCN-1, VAB-19, T05F1.4, and Y45F10D.13



5) ALX-1, D1081.7 (1J65), Y11D7A.12, and ALH-9



6) ALH-9, Y37E3.11, XH423, and F23B12.5 (5O926)



7) ALX-1, Y37E3.11 (1C653), Y11D7A.12, and ALH-9

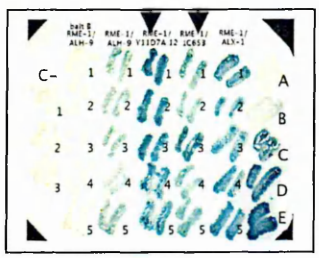


Figure 23 Reps-1 Criss-Cross Transformation:

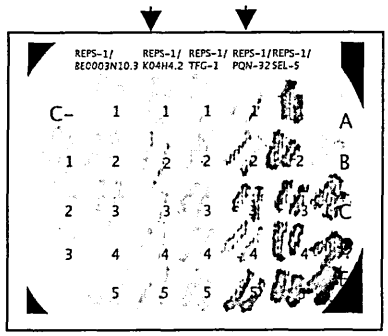
The X-gal Assays of clones that were transformed with Reps-1 Bait C and the cDNAs of the putative interactors that were identified from the Y2H screenings of ITSN-1, EHS-1, and RME-1.
A,B,C,D,E: Yeast Control strains transformed with plasmids containing no inserts (A), or transformed with plasmids expressing interactors of increasing interaction strengths in the order of B-E, as described in detail in Table 1 in Section 6.2.3.

Three yeast clones co-transformed with the bait construct pDBLeu-REPS-1 and the empty prey vector pPC86 were used as the negative control, as well as the control strain A.
1,2,3,4,5: Five different co-transformants tested in order to ensure that the *LacZ* activation was not observed not only in one clone but in multiple clones.
Five co-transformants of the REPS-1 bait and the prey expressing SEL-5, a putative interactor of REPS-1 previously identified and confirmed in the Y2H screenings, were used as the positive control for each membrane.

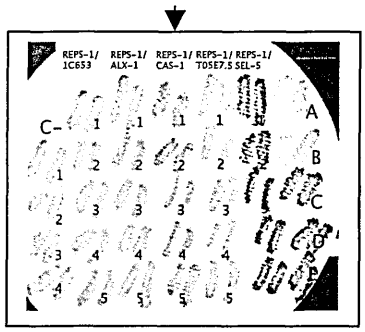
The scanned images of the membranes appear as if there was a strong background activation of *LacZ* gene, as the negative controls are also coloured. However, the colour of the negative control clones during a total of 24 hours for the X-gal assay was equivalent to that shown by the negative control clones in the membranes from the Criss-Cross Transformation of ITSN-1. The difference in the colour of the clones in the membranes in this assay is thought to be due to the fact that the membranes were not dried well when they were stored prior to the scanning of the images.

The positive interactions are indicated with arrows.

1) SEL-5, PQN-32, TFG-1, K04H4.2, and BE0003N10.3



2) SEL-5, T05E7.5, CAS-1, ALX-1, and Y37E3.11 (1C653)



3) SEL-5, Y45F10D.13, Y11D7A.12, and C50C3.8

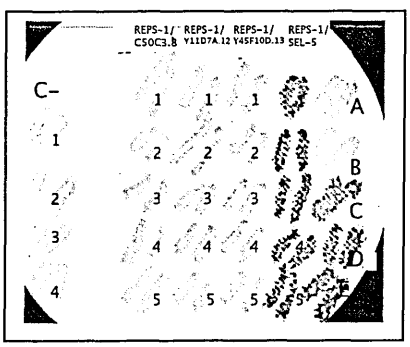


Figure 24. R10E11.6 Criss-Cross Transformation:

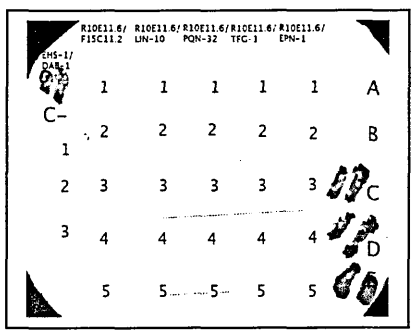
The X-gal Assays of clones that were transformed with R10E11.6 Bait B and the cDNAs of the putative interactors that were identified from the Y2H screenings of ITSN-1, EHS-1, RME-1, and REPS-1.

A,B,C,D,E: Yeast Control strains transformed with plasmids containing no inserts (A), or transformed with plasmids expressing interactors of increasing interaction strengths in the order of B-E, as described in detail in Table 1 in Section 6.2.3.

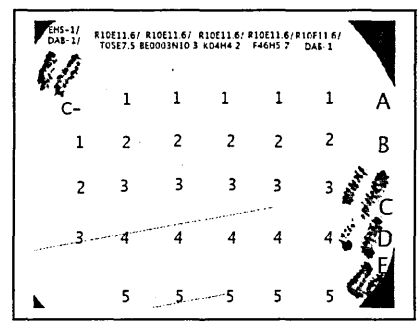
1,2,3,4,5: Five different co-transformants tested in order to ensure that the self-activation was not observed not only in one clone but in multiple clones.

Re-transformation of EHS-1 with DAB-1 was used as a positive control for the X-gal assay of each membrane, streaked above the negative control. Re-transformation of R10E11.6 bait with SEL-5, PCN-1, VAB-19 and F23B12.5 did not give any colonies and were not tested.

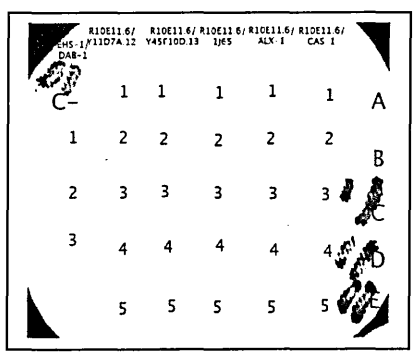
1) EPN-1, LIN-10, PQN-32, TFG-1, and F15C11.2



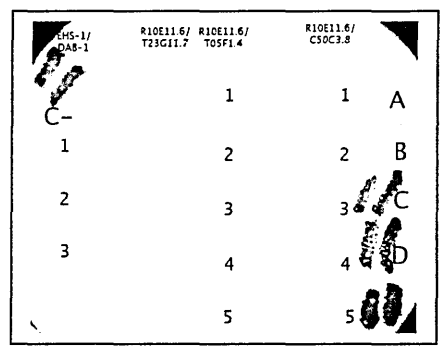
2) DAB-1, F46H5.7, K04H4.2, BE0003N10.3, and T05E7.5



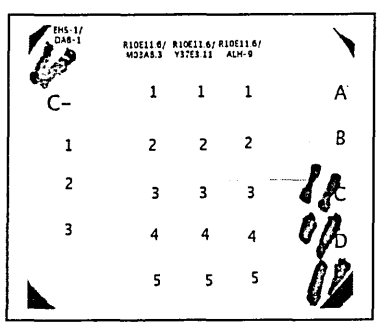
3) CAS-1, ALX-1, D1081.7 (1J65), Y45F10D.13, and Y11D7A.12



4) C50C3.8, T05F1.4, and T23G11.7



5) ALH-9, Y37E3.11 and M03A8.3,



The EH proteins, for which several bait constructs had been used in Y2H screenings, such as EHS-1 and RME-1, were tested by criss-cross transformation using only one of the baits. Bait 123 was used for EHS-1 rather than Bait 125, for two main reasons. Firstly, Bait 123 encodes a more complete EH domain-containing regions of EHS-1, as it contains first and the second EH domain whereas Bait 125 contains only the third EH domain. Secondly, the Y2H screenings carried out with Bait 125 did not identify any proteins that had not been found by the screenings with Bait 123.

Table 11 The interactions identified in Y2H screenings and by the criss-cross transformations

	Protein	INT-1	EHS-1	REPS-1	RME-1
1	EPN-1	+	+	+	-
2	LIN-10	+	+	+	-
3	PQN-32	+	+	+	-
4	TFG-1	+	+	-	-
5	F15C11.2	+	+	+	-
6	DAB-1	+	+	+	-
7	F46H7.5	+	+	-	-
8	K04H4.2	+	+	+	-
9	BE0003N10.3	+	-	-	-
10	T05E7.5	+	+	-	-
11	CAS-1	+	+	+	-
12	ALX-1	+	+	-	+
13	D1081.7	-	+	-	-
14	Y45F10D.13	-	+	-	-
15	Y11D7A.12	-	+	-	+
16	C50C3.8	-	+	-	-
17	B0041.2	+	+	+	-
18	T05F1.4	-	+	+	-
19	SEL-5	-	-	+	-
20	PCN-1	-	-	+	-
21	VAB-19	-	-	+	-
22	ALH-9	-	-	+	-
23	Y37E3.11	-	-	+	+
24	M03A8.3	-	-	+	-
25	F23B12.5	-	-	+	-
26	T23G11.7	-	-	+	-

+	Found positive in the Y2H screenings and confirmed by re-transformation assays
+	Newly Found positive by the criss-cross transformations for the *LacZ* expression
-	Found negative in the criss-cross transformation for the *LacZ* expression

Y2H screenings for RME-1 interactors were carried out using bait constructs D, F and H. Bait F was considered to be unsuitable for criss-cross transformation, as the bait alone was able to self-activate the expression of *LacZ* and required a tight monitoring of the reaction time for the X-gal assay. Similarly, Bait H was could self-activate *URA3*. Despite the fact that only *LacZ* expression was monitored in the criss-cross transformations, construct H was not used in order to reduce the risk of false positives. Although Bait D may indicate a restricted range of interactions mediated by the EH domains of RME-1, it was considered as the best out of all constructs available, as it had been shown to bind to an NPF-containing protein, ALX-1, which was also the only protein repeatedly confirmed to interact with RME-1 EH domain. Construct J, which was used for a re-transformation assay, or a whole-length RME-1 were not used in criss-cross transformation in order to be specific to the interactions mediated by the EH domain, and not to include baits with regions such as coiled-coil domain that may provide other interaction surfaces.

The interactions that were identified in the Y2H screenings and in the criss-cross transformation assays are summarized in Table 11 and in the Y2H column in Table 12.

Table 12 The summary of interactions found between a total of 26 proteins identified by the Y2H screenings and the 4 EH proteins: ITSN-1, EHS-1, Reps-1 and RME-1.

Gene Names of putative interactors	Bind. motif	ITSN-1			EHS-1			REPS1			RME-1			
		Y2H with INT-1	IVB with Full-length ITSN-1	genetic interaction by RNAi?	Y2H with EHS-1	IVB with Full-length EHS-1	genetic interaction by RNAi?	Y2H with Reps1	IVB with Full length REPS1	genetic interaction by RNAi?	Y2H with RME-1	IVB with Full-length RME-1	IVB with FL RME-1, with mutated EH domain	genetic interaction by RNAi?
EPN-1	4 NPF	++++	+++	-	++++	++	+	+++	-	+	-	++	-	-
LIN-10	2 NPF	++	++	+	++++	++++	+	+++	-	-	-	+++++	+++	-
DAB-1	2 NPF	+	+++	+	+++	+++++	+	+++	-	+	-	+++++	+++	+
F15C11.2 (1H603)	2 NPF	+	+	+	++	+/-	+	++++	-	+	-	+/-	-	-
ALX-1	1 NPF	+*	++	-	+	+	-	-	-	-	+	+	+/-	-
CAS-1	1NPF	+*	+++	-	+	+++	-	+*	-	-	-	+++	++	-
Y45F10D.13 (4O533)	1HSF	-	++	-	+	+++++	+	-	-	-	-	-	-	-
Y11D7A.12 (4K28)	1 FWR	-	+	+	+	++++	+	-	-	-	+*	+	+/-	+
C50C3.8 (3J252)	None	-	+++	+	+	+/-	+	-	-	-	-	++++	+++	+
BE0003N10 .3 (3C136)	None / NPW / NPY	+	+++	-	-	+/-	-	-	-	+	-	++++	+++	-
T05E7.5 (1G778)	1 NPF	++	+++	-	+*	++	-	-	-	+	-	+++++	+++++	-
PQN-32	2 NPF	++	++	-	++	+	-	+*	-	+	-	++	-	-
TFG-1	1NPF	+	+	-	++++	+/-	+	-	-	+	-	-	-	-
F46H5.7 (XH858)	1NPF / 1 WW	+	N/A	-	++	N/A	+	-	N/A	-	-	N/A	N/A	+
B0041.2 (1F227)	1 NPF	+*	N/A	-	+	N/A	-	+	N/A	+	-	N/A	N/A	-
D1081.7 (1J65)	1 HTF/ 1 FWR	-	N/A	-	++++	N/A	+	-	N/A	-	-	N/A	N/A	-
K04H4.2 (3K438)	1 NPF	+	+	-	+*	++	+	+*	-	+	-	++++	+++	-
T05F1.4 (1K209)	1 NPF	-	++	-	-	++	+	++	-	-	-	+++	+	-
sel-5	1 NPF / 1 HSF	-	+/-	+	-	+	-	++	-	+	-	-	-	-
pcn-1	None	-	-	+	-	-	-	+	-	-	-	-	-	+
vab-19	None	-	N/A	-	-	N/A	-	+++	N/A	+	-	N/A	N/A	+
alh-9 (F01F1.6)	None	-	N/A	+	-	N/A	+	+	N/A	+	-	N/A	N/A	-
Y37E3.11 (1C653)	1 DPF	-	N/A	-	-	N/A	+	+	N/A	-	+*	N/A	N/A	-
M03A8.3 (XH423)	None	-	N/A	+	-	N/A	-	+	N/A	-	-	N/A	N/A	-
F23B12.5 (5O926)	None	-	N/A	-	-	N/A	+	+	N/A	-	-	N/A	N/A	-
T23G11.7 (1I257)	None	-	-	-	-	-	+	+	-	-	-	-	-	-

: 13 interactions confirmed by Y2H, IVB and genetic assays

: 12 physical interactions confirmed (Y2H and IVB)

: 16 interactions confirmed physical and genetic (Y2H or IVB, and genetic)

The levels of expression were compared with that of the control strains A-E as follows:

A:-, B: +, C: ++, D:+++, E: ++++

A,B,C,D,E: Yeast Control strains transformed with plasmids containing no inserts (A), or transformed with plasmids expressing interactors of increasing interaction strengths in the order of B-E, as described in detail in Table 1 in Section 6.2.3.

9. Representation of cDNAs encoding NPF-containing proteins in the cDNA library

The level of representation of genes encoding the NPF-containing proteins in the cDNA library was of interest because a few of the interactors identified in the screening were identified from multiple clones. Therefore, it was important to ensure that there was no bias in the cDNA library used during the screening that could have affected the frequency of the cDNAs identified.

A total of 16 proteins were selected for the qPCR analysis. 4 proteins were chosen for 4 groups of proteins using the following criteria. The first group consists of NPF-containing proteins that had been identified from the Y2H screenings with high frequencies. The second group consists of the NPF-containing proteins that were identified once in EHS-1 screening. The third and the fourth groups represent random *C. elegans* proteins containing multiple NPF motifs, or a single NPF-motif, respectively. They were checked for their presence in the cDNA library in order to indicate that there was no bias in the screening by the skewed representation of the library. After the set-up of the conditions and testing the specificity of the pimers, 3 genes were removed and a total of 13 genes were quantified by QPCR. There are a total of 863 genes that encode amino acid sequences containing at least one NPF motif [18]. 13 genes out of 863 genes account only for 1.5% of the total number of genes encoding NPF motifs. Therefore, this QPCR analysis was carried out with the aim of indicating whether there is a correlation between the number of clones from which a specific NPF-encoding cDNA is isolated (frequency) and the level of cDNA present in the library. The genes encoding the NPF-containing proteins selected are summarized in Table 13, with the arrows indicating the three genes that were discarded for the QPCR analysis.

Table 13 Sixteen genes encoding NPF-containing proteins selected for QPCR on cDNA library used in the Y2H screenings.

No.of NPF refers to the number of NPF motifs present in the primary sequence of the genes.

Frequency refers to the umber of clones from which the cDNAs encoding the gene of interest were extracted.

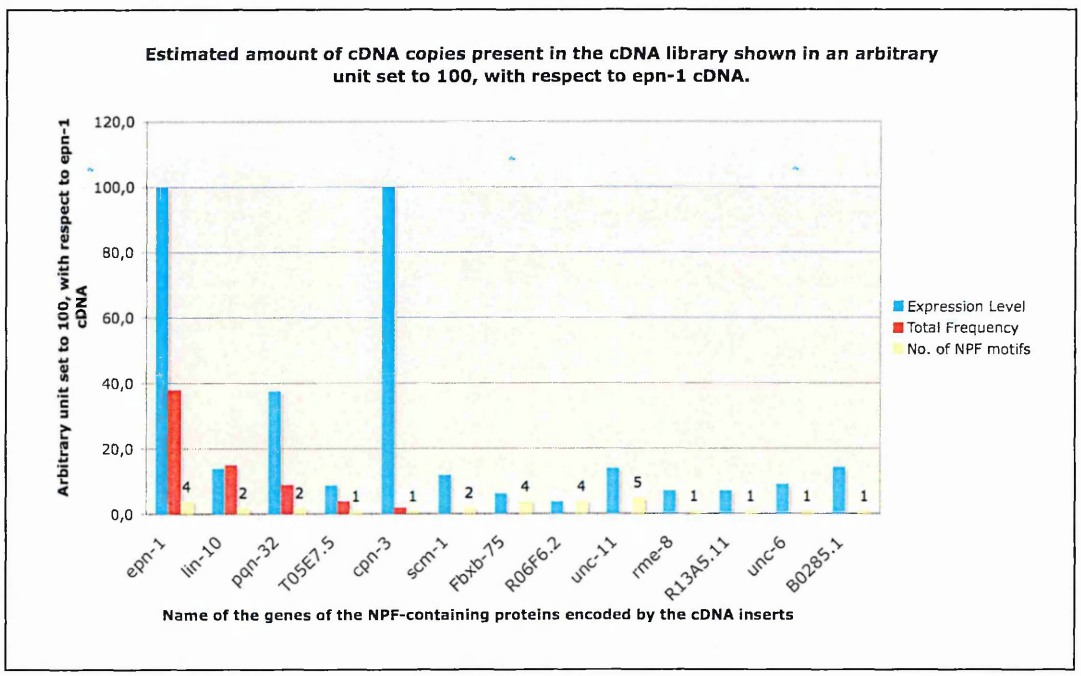
Genes	No. of NPF	Frequency
Epn-1	4	High
Lin-10	2	High
Pqn-32	2	High
Tfg-1	1	High
Cas-1	1	Low
Alx-1	1	Low
T05E7.5	1	Low
Cpn-3	1	Low
Scm-1	2	Never
Fbxb-75 (4F204)	4	Never
R06F6.2 (2L78)	4	Never
Unc-11	5	Never
Rme-8	1	Never
R13A5.11 (3I680)	1	Never
Unc-26	1	Never
B0285.1 (3F429)	1	Never

A correlation between the frequency with which a cDNA encoding a protein was identified in the Y2H screenings and the level at which it is represented in the cDNA library was found only for EPN-1, whilst for the other proteins, no such correlation was found. A group of NPF-containing proteins were selected from the *C. elegans* genome, representing some that were identified from several clones during the Y2H screenings in this study, and some that were never identified in the screenings. The qPCR amplification of these NPF-containing proteins suggested that the frequency with which the NPF-containing proteins were identified in the screenings is not dependent on the level of the cDNAs present in the library, with the exception of one gene, *epn-1* (Figure 25). The qPCR results also suggest that the whole genome had been covered by the cDNA library used in the screenings, because a small percentage (1.5%) of the genes

encoding NPF-containing proteins in the *C. elegans* genome that were chosen were all detected in the cDNA library.

Figure 25 A graph to show the quantification of the cDNAs encoding NPF-containing proteins amplified from the cDNA library by qPCR.

The expression level: 13 genes whose expression levels were detected by the qPCR (blue). Total Frequency: the number of clones from which the cDNAs encoding the specified gene was identified during the Y2H screenings (red).No. of NPF motifs: the numbers indicate the number of NPF motifs present in the amino acid sequence encoded by the specified gene.



As a further proof-of-principle of the Y2H screenings, UNC-26, a *C. elegans* homologue of Synaptojanin that interacts with mammalian Eps15 and Intersectin, was cloned in-frame with GAL4AD and tested for interaction with ITSN-1, EHS-1, RME-1 and REPS-1 by re-transformation assays. None of the re-transformed clones showed *LacZ* expression (results not shown). Furthermore, the cDNA encoding SCM-1 was also cloned in-frame with the GAL4AD, and it was checked for its putative interaction with R10E11.6, but no interaction was found. These results, showing that the theoretical interactors of *C. elegans* EH proteins that were not identified in the Y2H screenings indeed do not to interact, support the accuracy of the Y2H screening.

10. GST Pull-down Assays

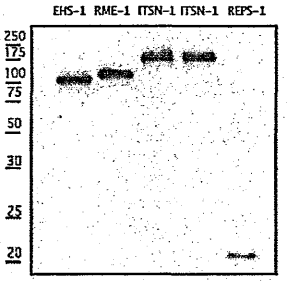
(These experiments were carried out by Dr. Francesca Senic-Matuglia, IFOM, Milan, Italy)

The putative interactions identified by Y2H were validated by *in vitro* pull-down assays. In the first round of pull-down assays, 18 out of 26 interactors were selected to represent a range of EH interacting motifs found in the amino acid sequences. 12 proteins contained multiple or single NPF motifs (Class I), 1 protein each for Class II (Y45F10D.13) and Class III (Y11D7A.12) peptide motifs, and two proteins with no known interacting motifs, namely C50C3.8 and BE0003N10.3. The Y2H results indicated that BE0003N10.3 is an interactor specific to ITSN-1, whereas Y45F10D.13 and C50C3.8 are specific interactors of EHS-1. Therefore, not all of the eighteen proteins, which were cloned as GST-fusion proteins, interacted with all four EH proteins in Y2H. However, the pull-down assays were performed against all four EH proteins, in order to detect the interactions that were not detected by the "criss-cross" transformations.

The shortest cDNA identified in the screenings for each interactor was cloned in pGEX vector and was expressed as a GST-fusion protein. The *C. elegans* EH proteins were over-expressed as FLAG-tagged full-length proteins in Phoenix cells. The full-length proteins were used in order to provide the proteins in conformations that resemble the physiological conditions, and also to detect the interactions that are mediated by the EH domains but assisted by other regions of the EH proteins, especially in the case of RME-1, which will be discussed in the section 15.4. Anti-FLAG antibody was used to recognise the EH proteins as there are antibodies available only against ITSN-1 and EHS-1 and not against RME-1 and REPS-1. The amount of the EH proteins used in the pull-down assays were checked and were

controlled to be approximately equivalent to each other by anti-FLAG Western blot as shown in Figure. 26.

Figure 26. Anti-FLAG Western Blot to show the normalization of the quantities of FLAG-tagged EH proteins used in the GST Pull-down assays.



10.1 ITSN-1 Pull-down assay

As shown in Figure 27 and summarized in Table 12, 10 interactors of ITSN-1 that had been identified by Y2H screenings were confirmed by *in vitro* pull-down assay (Figure 27 Itsn1, Table 12). ITSN-1 recognised by anti-FLAG for the pull-down by F15C11.2 was identified only by a very weak signal, indicating that the strong interaction found in the Y2H system may not represent the actual interaction strength. In comparison, C50C3.8 and Y45F10D.13, which were thought to interact only with EHS-1 were demonstrated to interact at a level comparable to that of ALX-1 and LIN-10 (Figure 27 Itsn1, Table 12). In addition, Y11D7A.12 (4K28), which was found to interact with EHS-1 and RME-1 in the Y2H system, also interacted with full-length ITSN-1 by the *in vitro* pull-down assays (Figure 27 Itsn1, and the column for ITSN-1 *In Vitro* Binding (IVB) assays in Table 12).

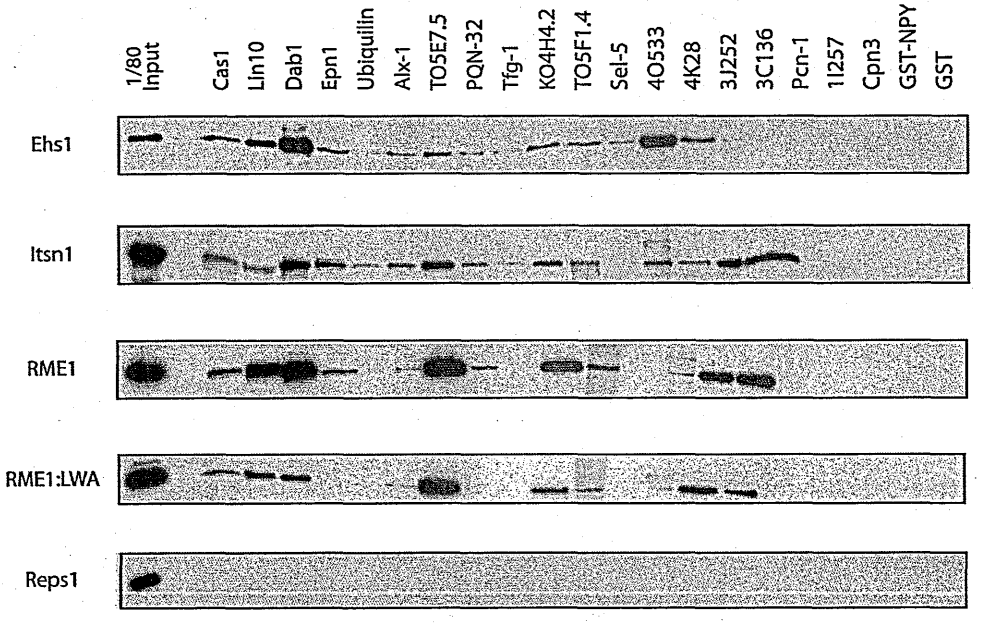
10.2 EHS-1 Pull-down assay

In vitro pull-down assays for EHS-1 validated 13 out of 16 interactors of EHS-1 that were identified by Y2H screenings. As it was seen for the interaction between ITSN-1 and F15C11.2, the latter interacted with EHS-1 with much less binding efficiency with respect to the other NPF-containing proteins, for example, CAS-1,

LIN-10 and DAB-1. (Figure 27 Ehs1, and the column for EHS-1 IVB assays in Table 12). Unlike ITSN-1, which interacted also with the other proteins that had not been identified previously in the Y2H system, EHS-1 did not interact with BE0003N10.3, which is an ITSN-1 specific interactor.

Figure 27 Anti-FLAG Western Blot of the pull-down assays:

18 putative interactors cloned as GST-fusion proteins: CAS-1, LIN-10, DAB-1, EPN-1, F15C11.2 (Ubiquilin), ALX-1, T05E7.5, PQN-32, TFG-1, K04H4.2, T05F1.4, SEL-5, Y45F10D.13 (4O533), Y11D7A.12 (4K28), C50C3.8 (3J252), BE0003N10.3 (3C136), PCN-1, T23G11.7 (1I257). 3 negative controls (CPN-3, GST-NPY peptide, and GST alone).
120 pmol of GST-fusion proteins were incubated with varying amount of total lysates of Phoenix cells over-expressing the FLAG-tagged EH proteins, in order to have equivalent amount of each FLAG-tagged EH proteins, as normalized by the anti-FLAG Western blot showin in Figure 26.



10.3 RME-1 Pull-down assay

Amongst the eighteen GST-fusion proteins tested by *in vitro* pull-down assays, there were two RME-1 interactors, which had been identified in Y2H screenings, and they were both confirmed by the pull-down assays (Figure 27 RME1 and the column for RME-1 IVB in Table 12).

In addition, ten proteins were identified to interact with the full-length RME-1 by the *in vitro* assays (Figure 27, RME1). Proteins such as CAS-1, LIN-10, DAB-1, EPN-1, T05E7.5, PQN-32, K04H4.2, and T05F1.4 are NPF-containing proteins, whereas C50C3.8 and BE0003N10.3 do not contain any known interaction motifs. The *in vitro* binding assays repeated with the mutated EH domain showed that the majority of these novel interactions were reduced but not disrupted. In comparison, three interactors, namely EPN-1, F15C11.2 (Ubiquilin) and PQN-32, were shown to be specifically mediated by the EH domain. In spite of the presence of an NPF-motif in its sequence, T05E7.5 was shown to interact with both constructs of full-length RME-1 with the comparable efficiency, indicating that it interacts with RME-1 at a region outside of the EH domain.

10.4 REPS-1 Pull-down assay

The expression of the FLAG-tagged REPS-1 was detected, although at a slightly lower level than the other EH proteins, no interactors were observed in the pull-down assays.

11. Genetic interactions 1: Aldicarb treatment of EH mutant strains

As discussed in the Introduction (Section 3.2.2), the mutant strain of *ehs-1 (ok146)* is hyposensitive [19] to aldicarb treatment, whereas *itsn-1* (*ok268*) is hypersensitive [63] to aldicarb, with respect to the response displayed by the *N2 Bristol* strain. The average length of time that the worms were responsive to touch was referred to as the average "survival" time in this analysis. The hypersensitive phenotype of *itsn-1* mutants demonstrated by the shorter average survival time (B) with respect to the wild type (N), in Figure 28a and 28b. Whereas the *ehs-1* mutant showed an aldicarb-hyposensitive phenotype, indicated by the longer average survival time for *ehs-1* (B) than that of the wild type (N) in Figure 29a and 29b.

In addition, a recently identified *reps-1* mutant strain was characterized in this study for its sensitivity to aldicarb. Under 0.5mM aldicarb, a batch of 40 *reps-1* mutants (B), repeated at least three times (n=40, in triplicates), have been found to be hypersensitive to aldicarb at a level similar to that of *itsn-1*, with respect to the wild type *N2 Bristol* strain (N) (Figure 30a and 30b). This observation is suggestive of a role of *reps-1*, which is still uncharacterized, in neurotransmission. The function of *reps-1* in synaptic transmission is currently under investigation.

In comparison, *rme-1 (b1045)* (B) showed no sensitivity to aldicarb, a phenotype very similar to that of the wild type (N), indicating that it does not have a phenotype related to aldicarb (Figure 31a and 31b). The mutant strain of *rme-1* has been reported to have a reduced brood size with respect to N2 [99]. The effect of knock-down of the putative interactors on the average brood size of *rme-1* mutants was also tested.

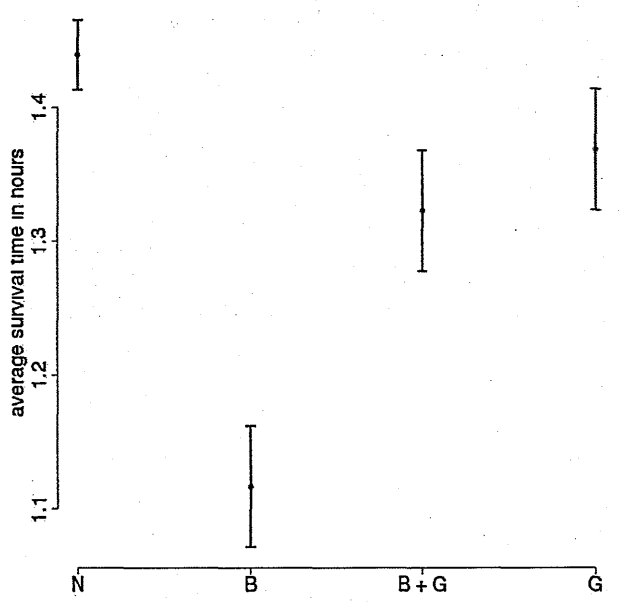
The mutant strain available for R10E11.6 (*ok619*) was not used, because the only mutant that is currently available also includes the deletion of the upstream gene, *vha-2*.

Figure 28. The average survival time (hours) under treatment with 0.5mM aldicarb of* itsn-1 *mutant worms in which 26 genes were knocked-down by RNAi. N2 Bristol (N), N2 (RNAi) (G), itsn-1 (B), and itsn-1 (RNAi) (B+G).

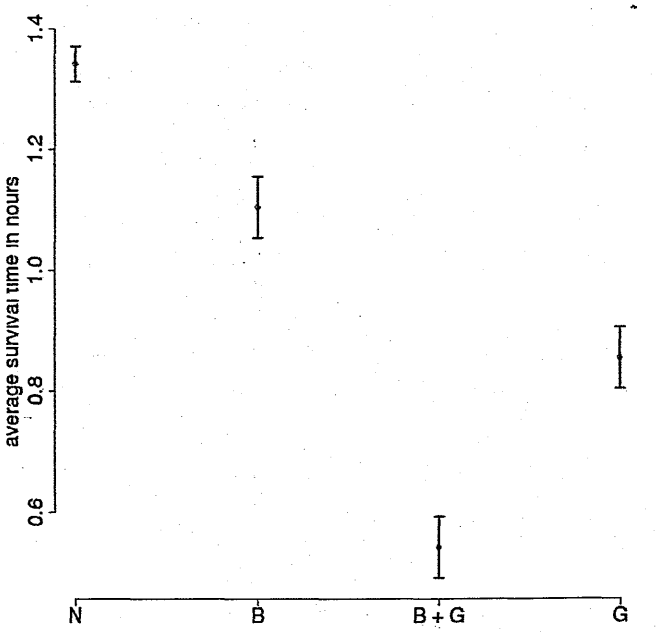
(n=40, in triplicates)
X-axis: The average survival time in hours under the treatment with 0.5mM aldicarb
Y-axis: N: wild type, B: itsn-1 mutant, B+G: itsn-1 mutant in which the specified gene was knocked down by RNAi, G: wild type in which the specified gene was knocked down by RNAi.

a. *itsn-1*: genetic interactions found

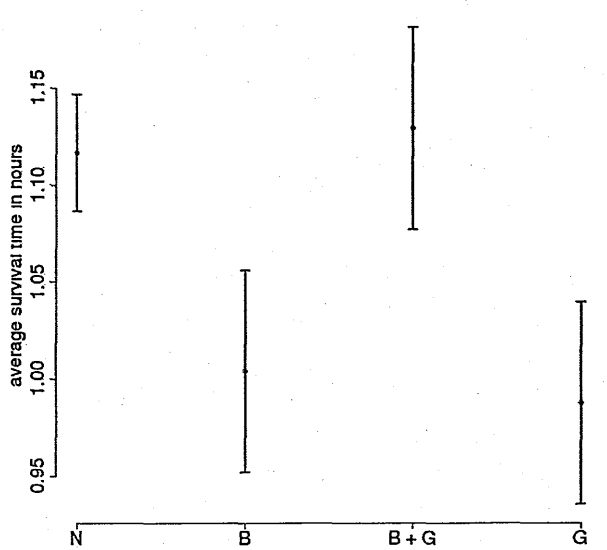
1) *itsn-1(RNAi:lin-10)*



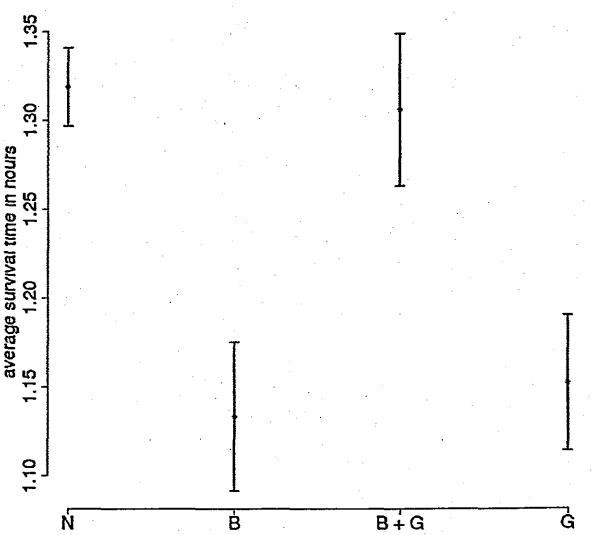
2) *itsn-1 (RNAi:dab-1)*



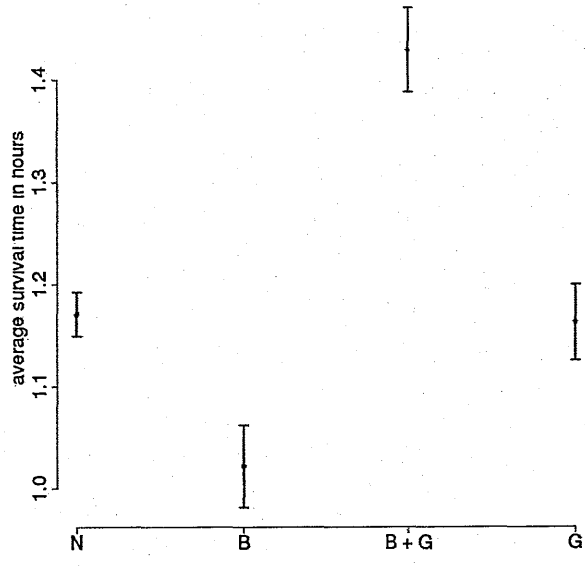
3) *itsn-1 (RNAi:F15C11.2)*



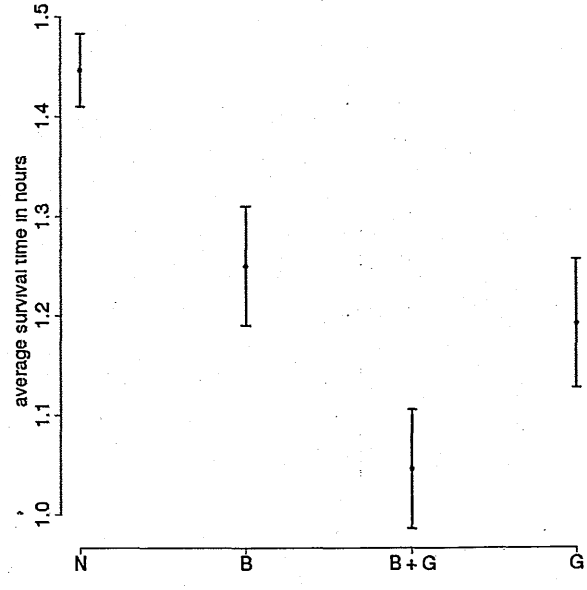
4) *itsn-1 (RNAi:C50C3.8)*



5) *itsn-1 (RNAi;Y11D7A.12)*

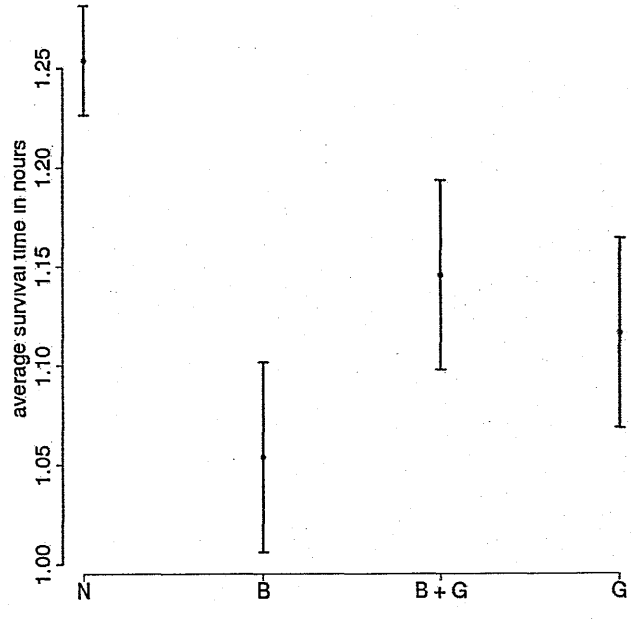


6) *itsn-1 (RNAi;pcn-1)*

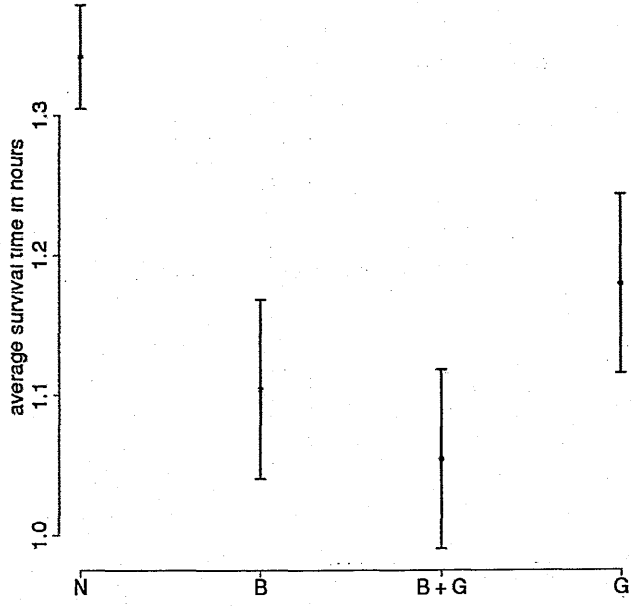


b. *itsn-1*: No genetic interaction found

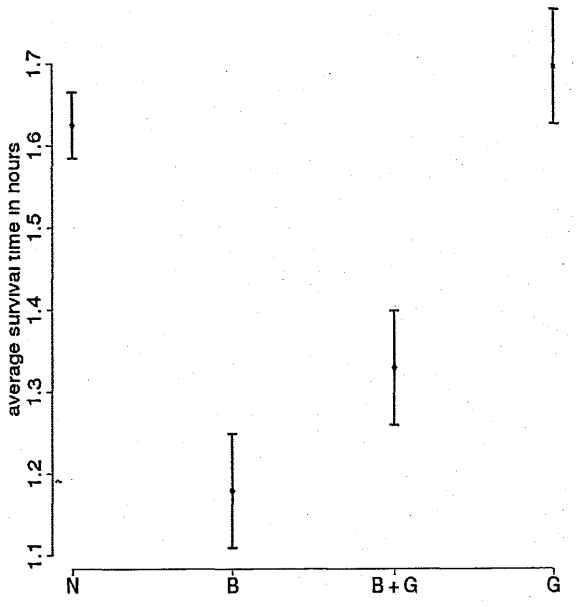
1) *itsn-1 (RNAi;B0041.2)*



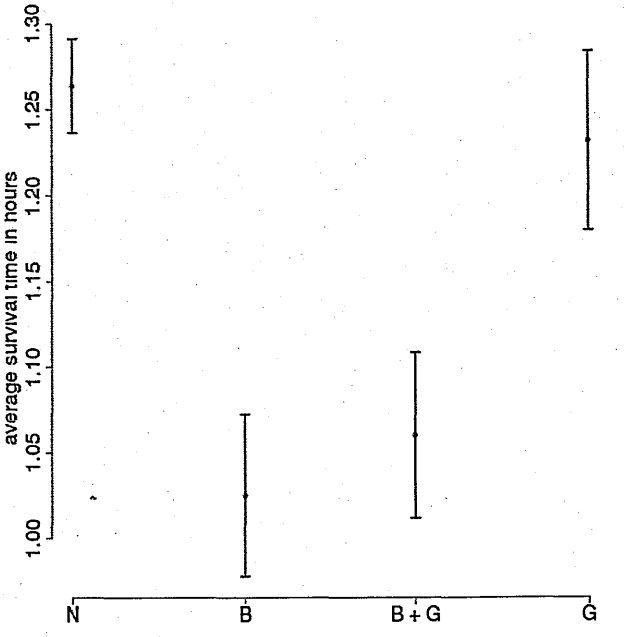
2) *itsn-1 (RNAi;cas-1)*



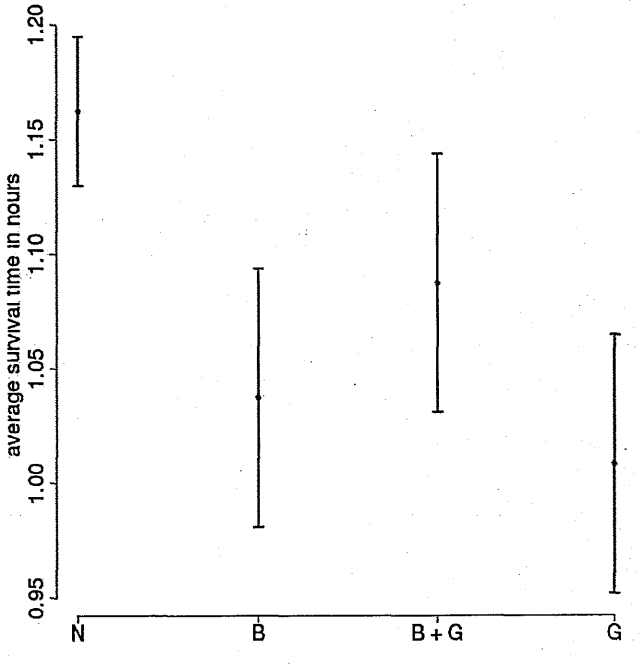
3) *itsn-1 (RNAi;epn-1)*



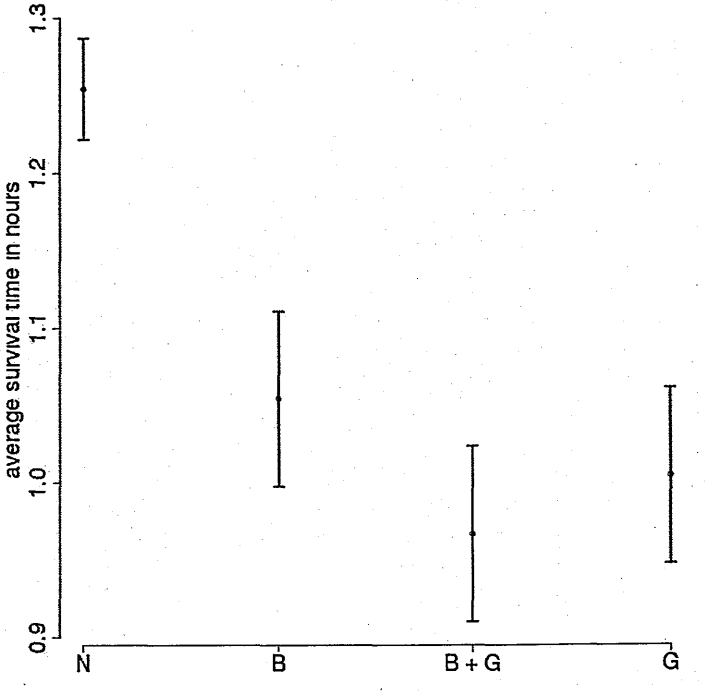
4) *itsn-1 (RNAi;F46H5.7)*



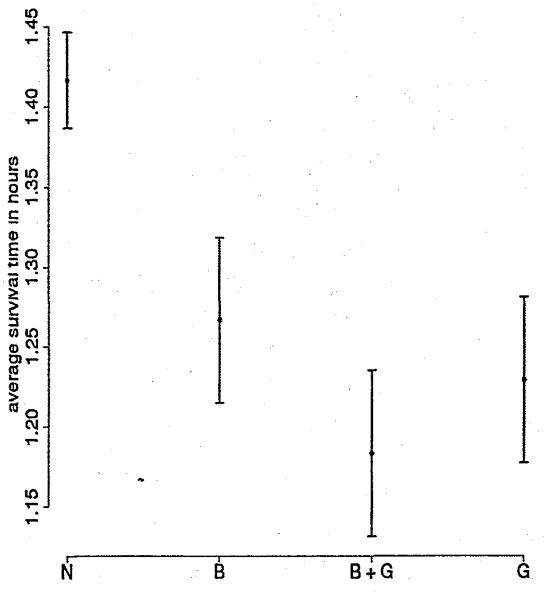
5) *itsn-1 (RNAi;K04H4.2)*



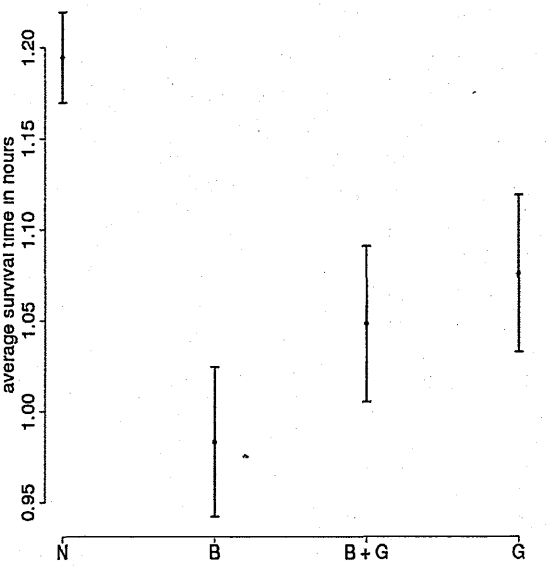
6) *itsn-1 (RNAi;pqn-32)*



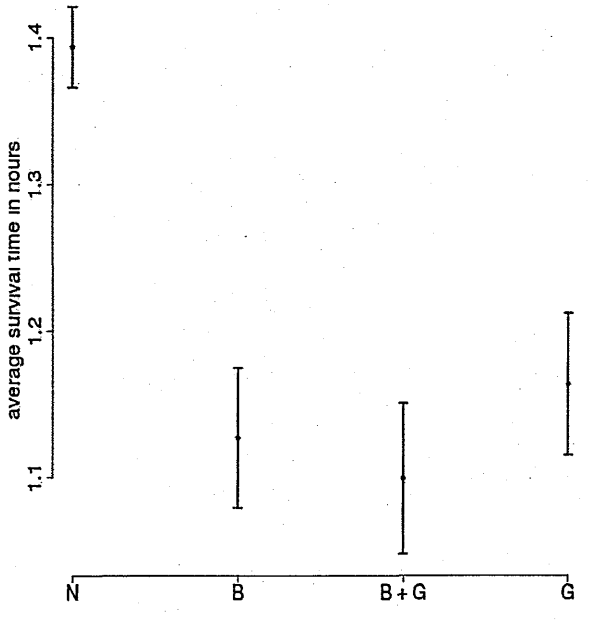
7) *itsn-1 (RNAi;T05E7.5)*



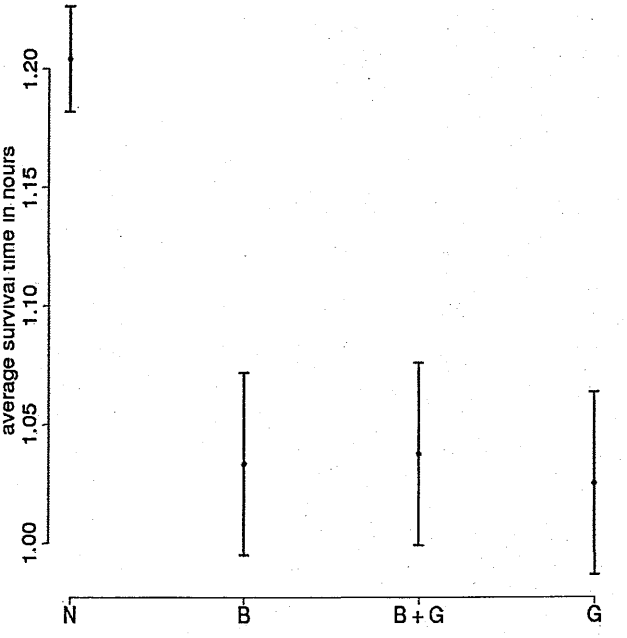
8) *itsn-1 (RNAi;T23G11.7)*



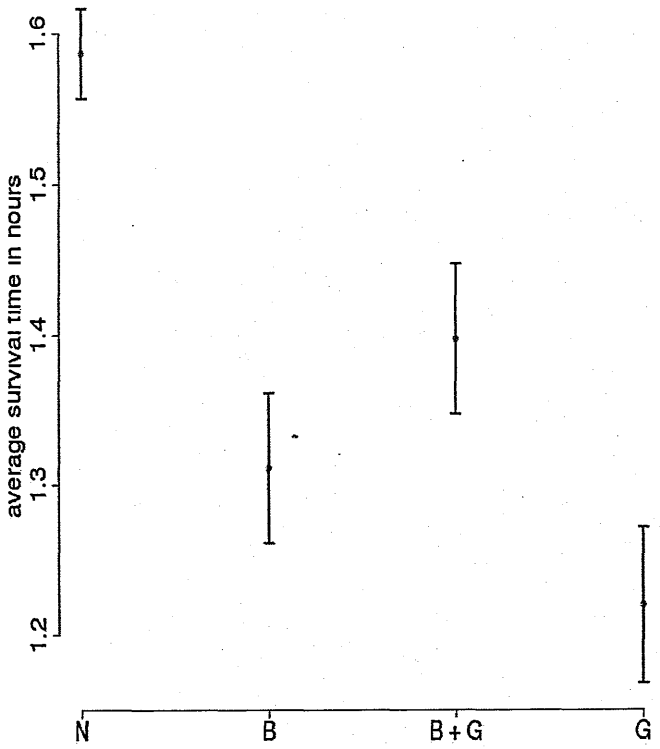
9) *itsn-1 (RNAi;tfg-1)*



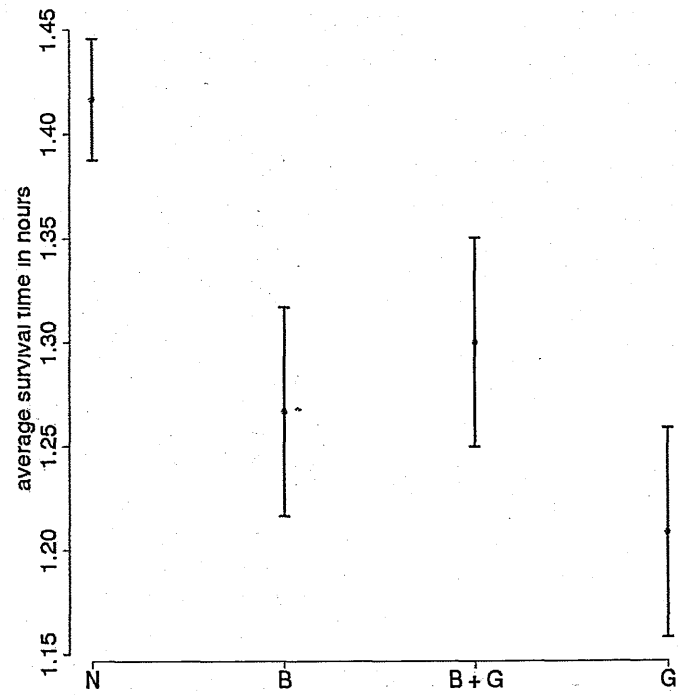
10) *itsn-1 (RNAi;vab-19)*



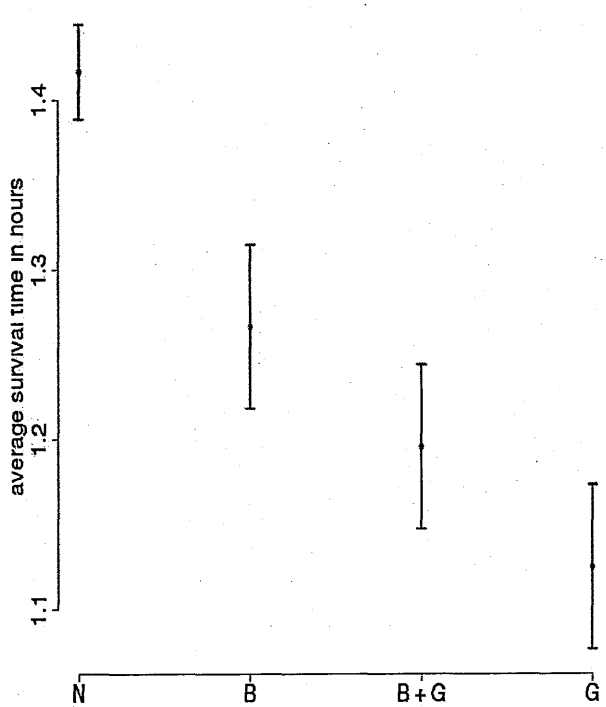
11) *itsn-1 (RNAi;Y45F10D.13)*



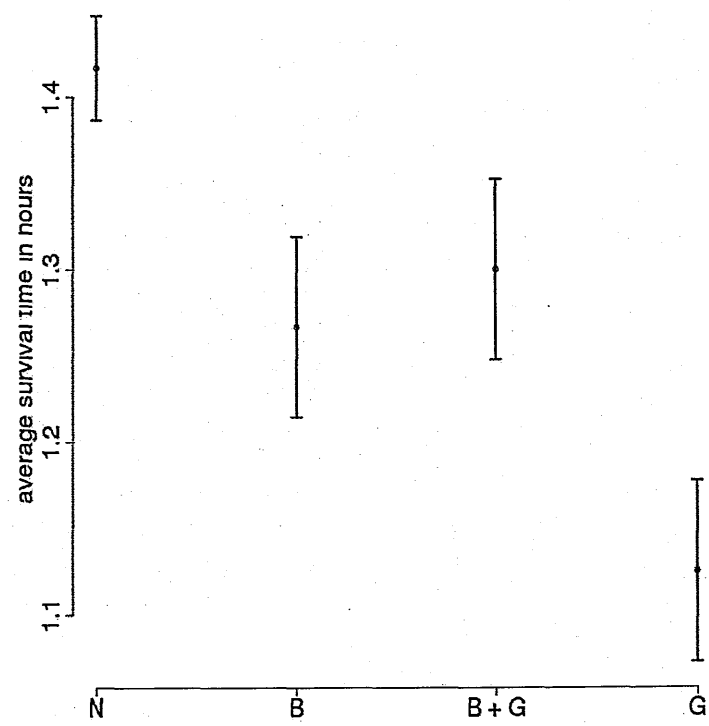
12) *itsn-1 (RNAi; Y37E3.11)*



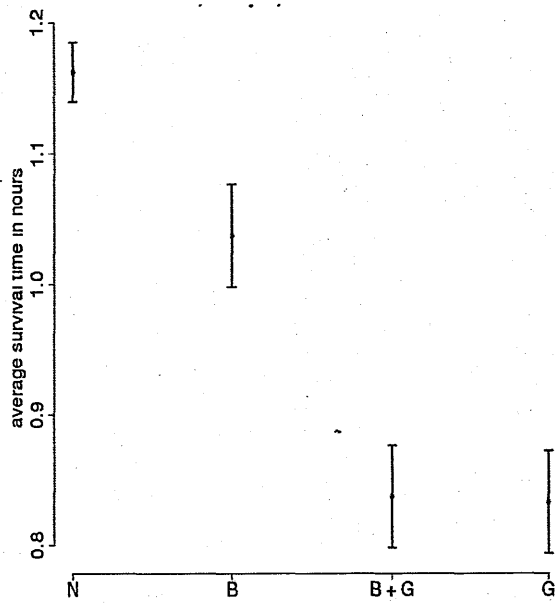
13) *itsn-1 (RNAi;F23B12.5)*



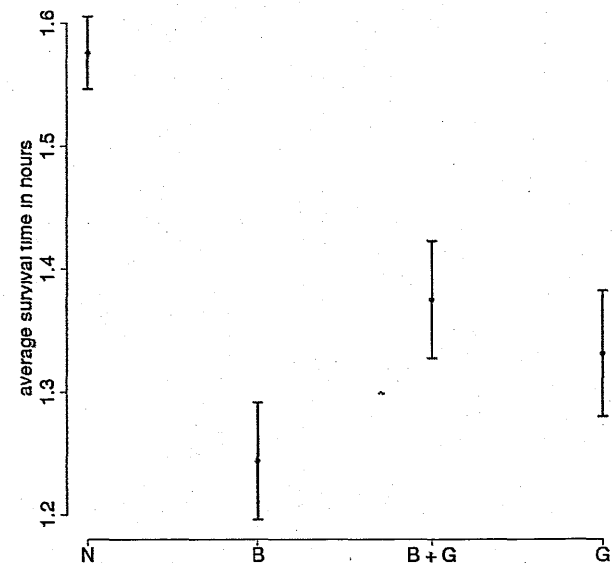
14) *itsn-1 (RNAi;T05F1.4)*



15) *itsn-1 (RNAi;D1081.7)*



16) *itsn-1 (RNAi;alx-1)*



17) *itsn-1 (RNAi;BE0003N10.3)*

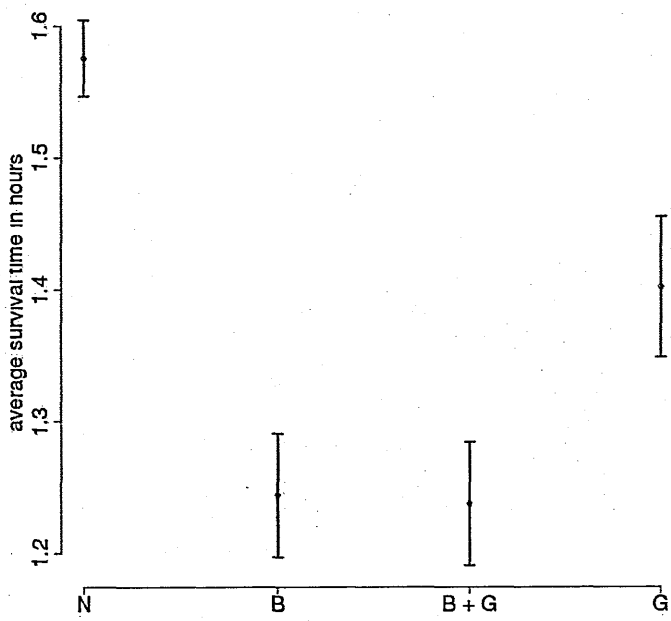
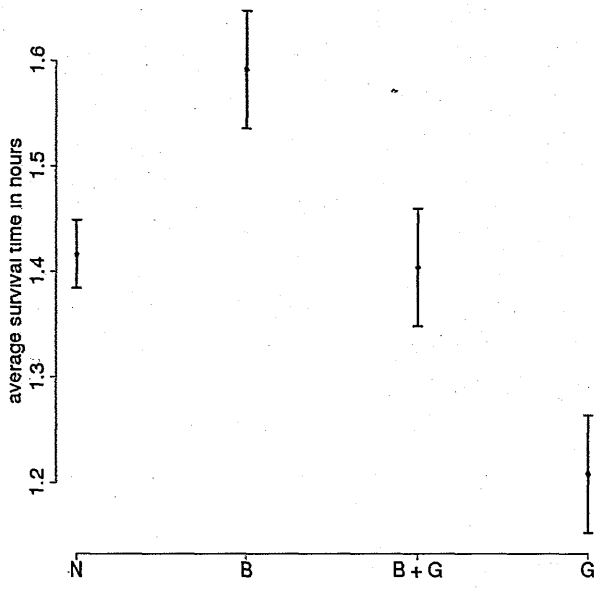


Figure 29. The average survival time under treatment with 0.5mM aldicarb of* ehs-1 *mutant worms in which 26 genes were knocked-down by RNAi.* N2 Bristol (N), N2 *(RNAi) (G),* ehs-1 (B), *and* ehs-1 *(RNAi) (B+G).

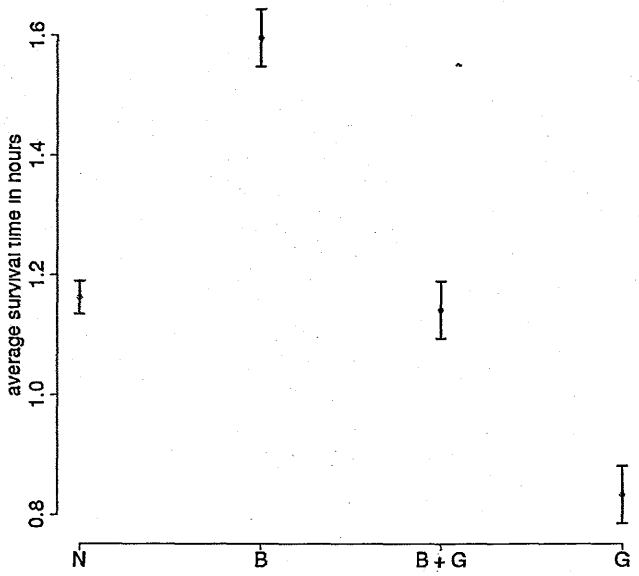
(n=40, in triplicates)
X-axis: The average survival time in hours under the treatment with 0.5mM aldicarb
Y-axis: N: wild type, B: itsn-1 mutant, B+G: itsn-1 mutant in which the specified gene was knocked down by RNAi, G: wild type in which the specified gene was knocked down by RNAi.

a. *ehs-1*: genetic interactions found

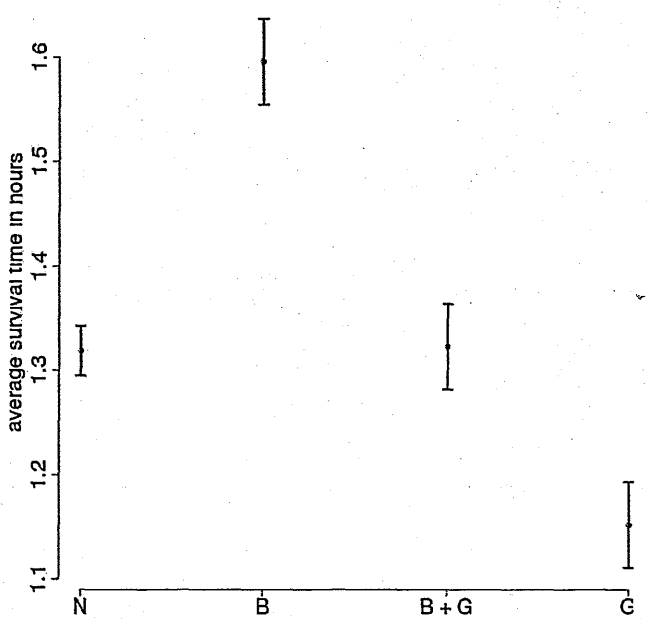
1) *ehs-1 (RNAi;Y37E3.11)*



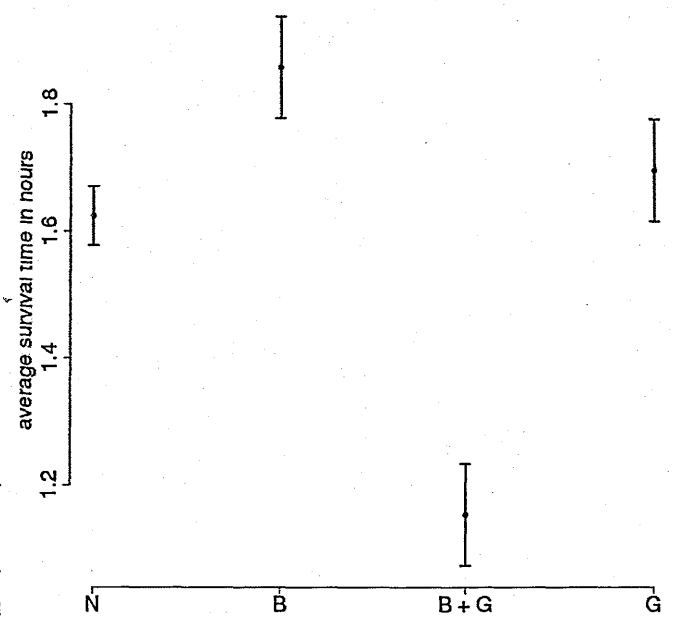
2) *ehs-1 (RNAi;D1081.7)*



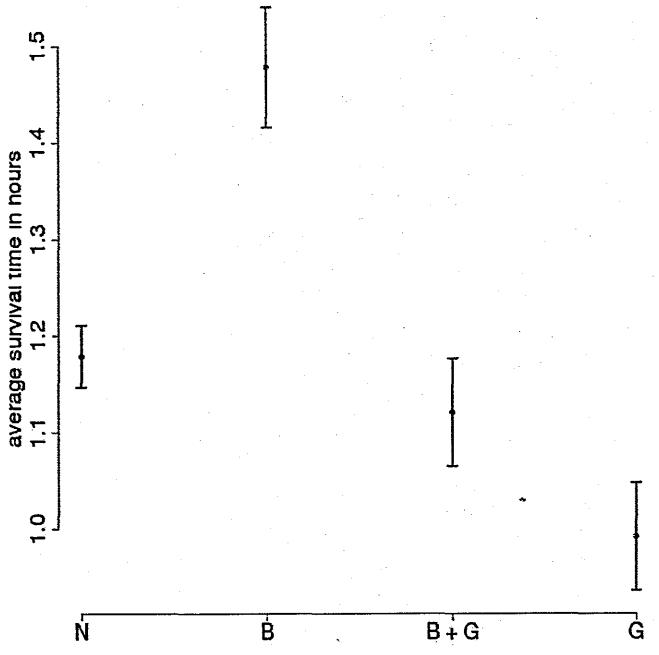
3) *ehs-1 (RNAi;C50C3.8)*



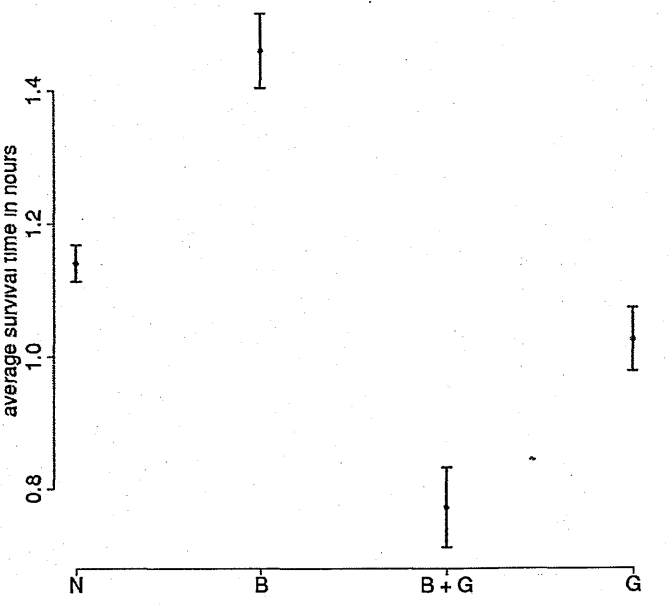
4) *ehs-1 (RNAi;epn-1)*



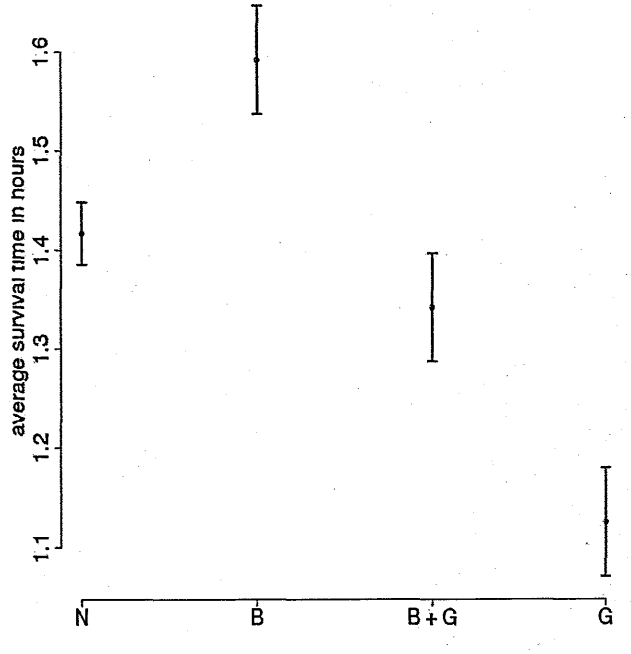
5) *ehs-1 (RNAi;F01F1.6)*



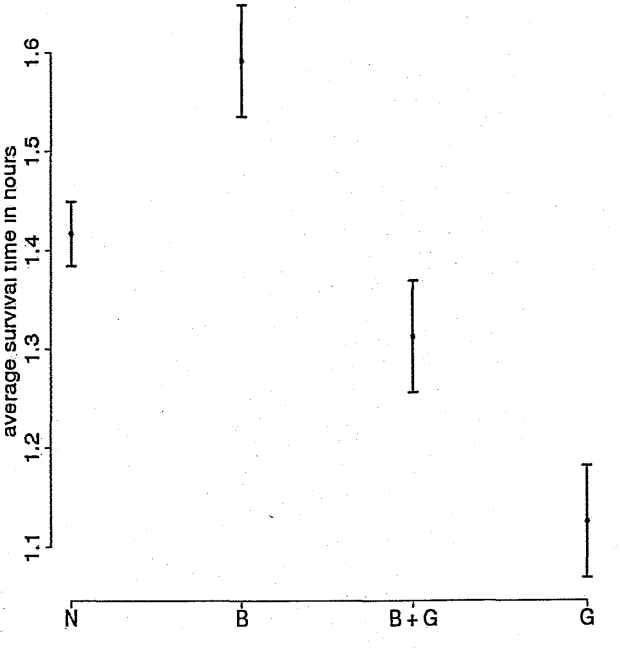
6) *ehs-1 (RNAi;F15C11.2)*



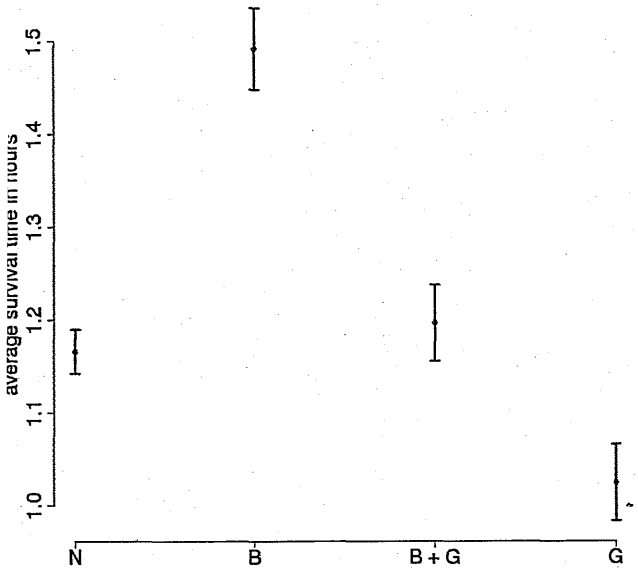
7) *ehs-1 (RNAi;F23B12.5)*



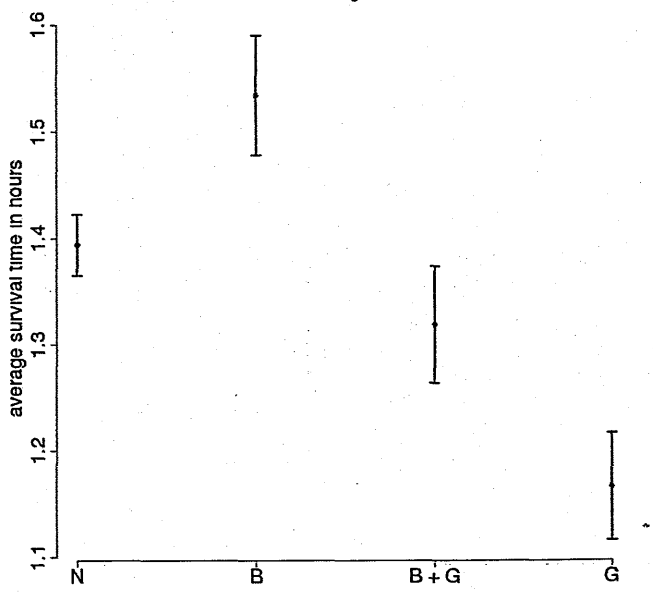
8) *ehs-1 (RNAi;T05F1.4)*



9) *ehs-1 (RNAi;T23G11.7)*

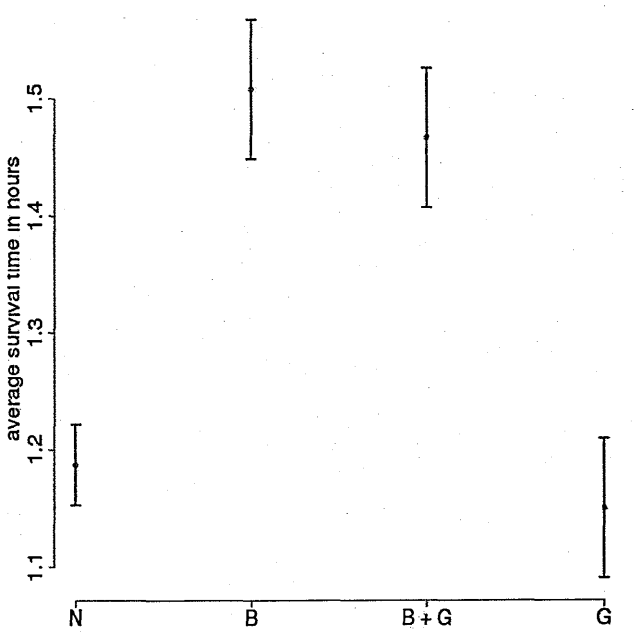


10) *ehs-1 (RNAi;tfg-1)*

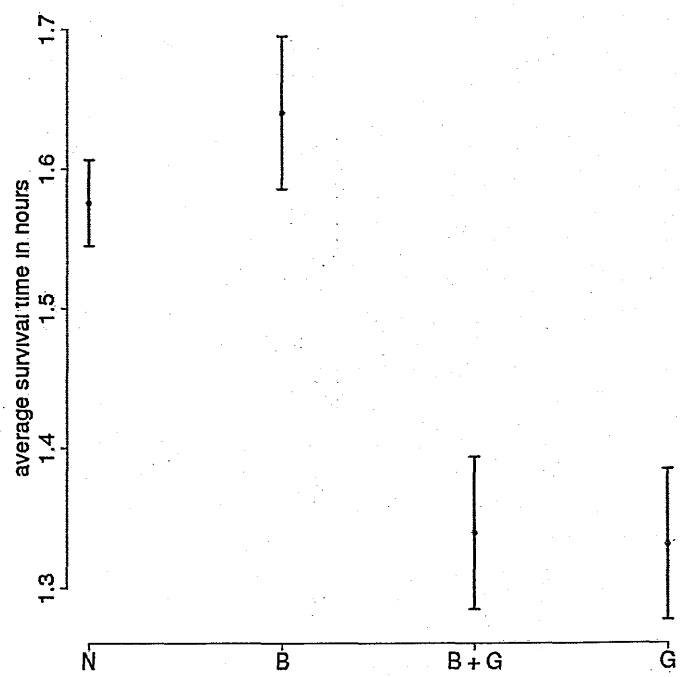


b. *ehs-1*: No genetic interaction found

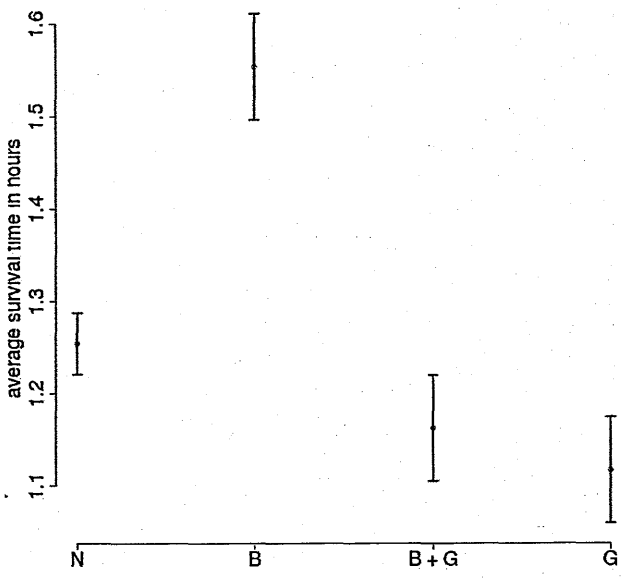
1) *ehs-1 (RNAi;M03A8.3)*



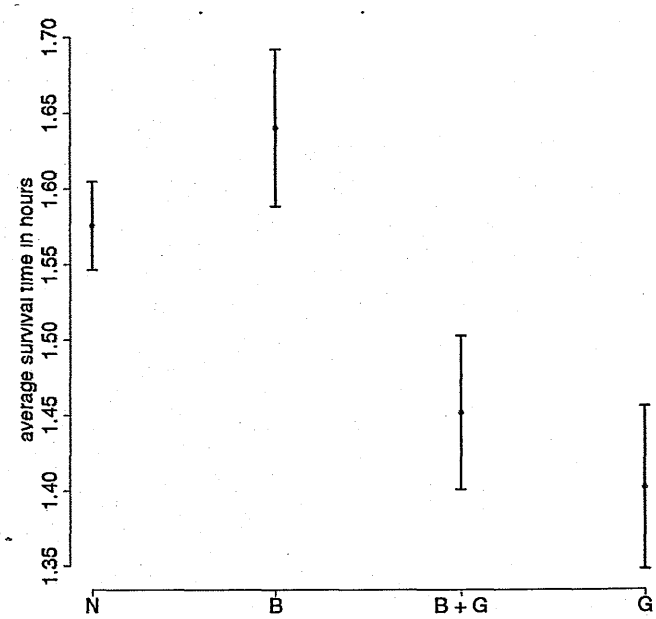
2) *ehs-1 (RNAi;alx-1)*



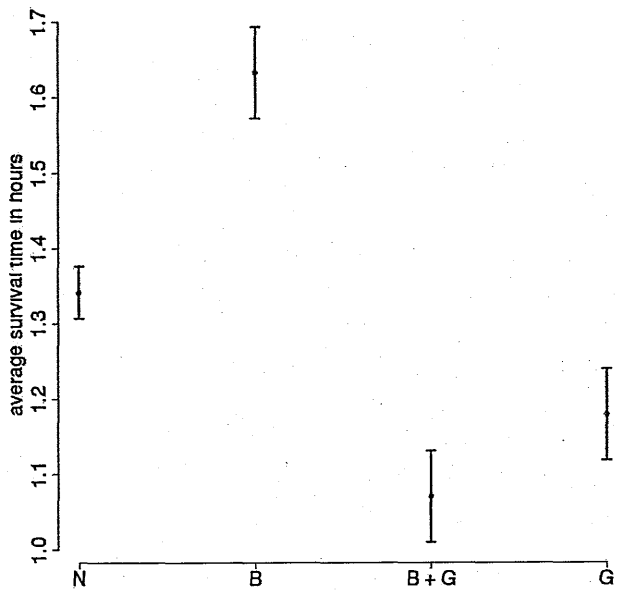
3) *ehs-1 (RNAi;B0041.2)*



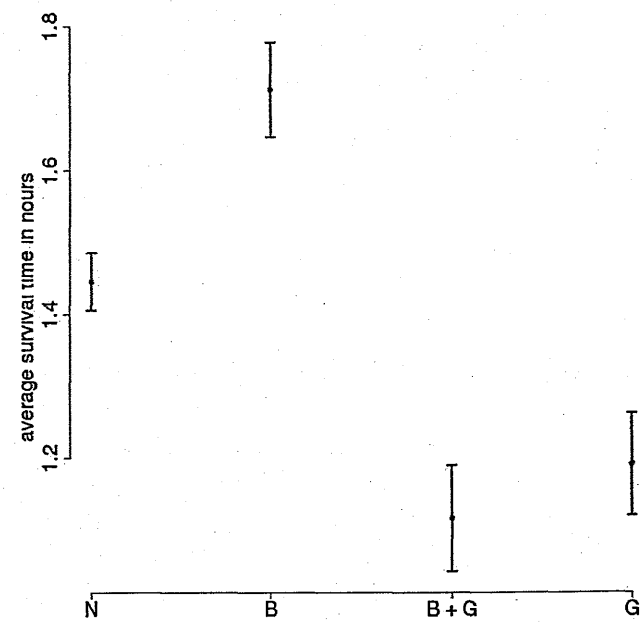
4) *ehs-1 (RNAi;BE0003N10.3)*



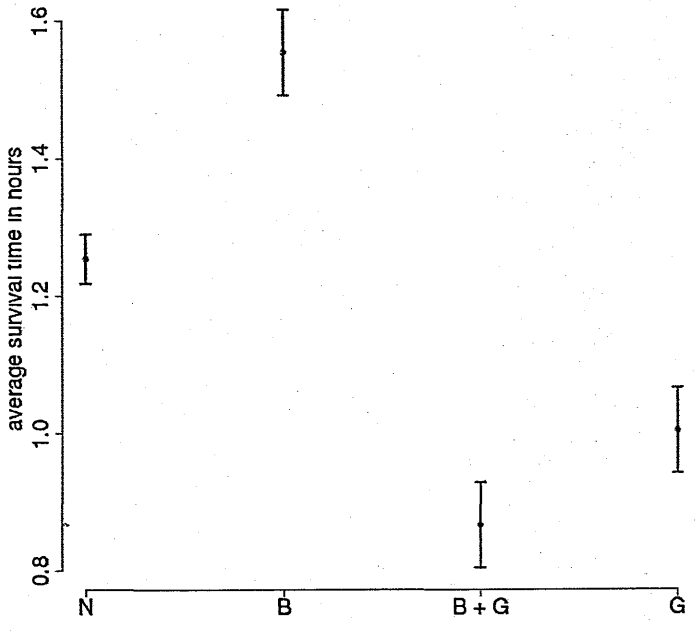
5) *ehs-1 (RNAi;cas-1)*



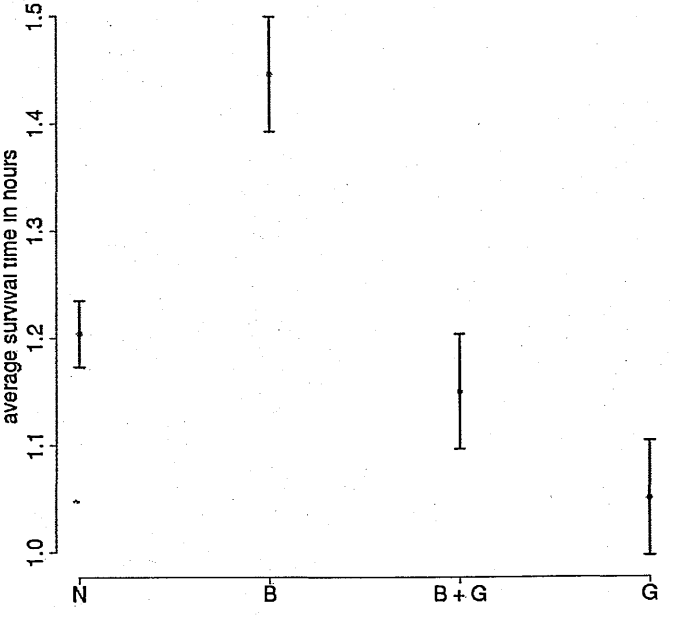
6) *ehs-1 (RNAi;pcn-1)*



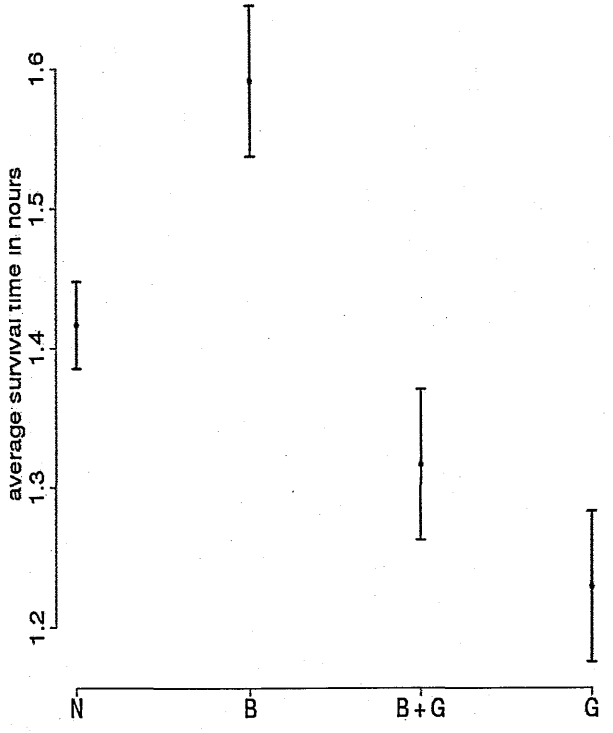
7) *ehs-1 (RNAi;pqn-32)*



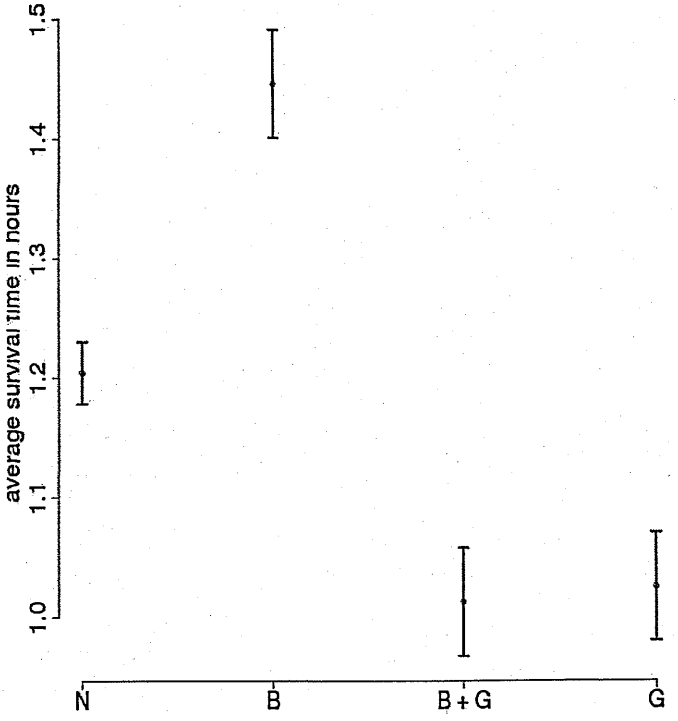
8) *ehs-1 (RNAi;sel-5)*



9) *ehs-1 (RNAi;T05E7.5)*



10) *ehs-1 (RNAi;vab-19)*



11.3 Assays for Touch Response under the Aldicarb Treatment

Each EH mutant strain was subjected to the knock-down of 26 genes in parallel with the *N2* worms. 60 young adults per strain were then placed on aldicarb plates containing 0.5mM aldicarb and the touch response by each worm was checked every 30 minutes for a total of three hours. The average time of "survival" when treated with 0.5mM aldicarb, meaning the average duration in hours that the worms were responsive to touch, was calculated for both *N2* and each EH mutant worms, in control condition and in the knock down. The average time of "survival" for *N2* fed with RNAi empty vector (N), for *N2* worm in which the putative interactor was knocked down (G), for the EH mutants fed with RNA*i* empty vector (B), and for the EH mutants in which the putative interactors were knocked down (B+G), were compared. When the effect of the knock down (G), had an effect on the average time of "survival" of the EH mutant genetic (B), such that the average time of "survival" of the knock-down EH mutant (B+G) was either extended or shortened, the effect was considered to indicate genetic interactions.

A total of 20 putative interactors out of 26 proteins found from the Y2H screenings of ITSN-1, EHS-1, REPS-1 and RME-1, were tested across all four respective EH mutants, even though some combinations had not been shown to interact physically by Y2H or by GST pull-down assays. The remaining 6 interactors were not tested because the preparation of the reagents required for RNAi specific for these genes failed.

11.3.1 *itsn-1* genetic interactions

Itsn-1 was found to genetically interact with a total of 9 genes; *lin-10*, *dab-1*, *F15C11.2*, *C50C3.8*, *Y11D7A.12*, *pcn-1*, *sel-5*, *F01F1.6*, and *M03A8.3* (Figure 28a). No interaction was determined for the cases of *F23B12.5* and *T05F1.4*, but the phenotype

of *itsn-1* mutants appeared to be more dominant over that of the RNA-interfered worms (Figure 28b).

11.3.2 *ehs-1* genetic interactions

The aldicarb experiments indicated that *ehs-1* could interact genetically with 16 genes. The plots of the average "survival" time for 15 interactions are shown in Figure 29a. The fifteen genes that were identified to interact with *ehs-1* are: *Y37E3.11*, *D1081.7 (1J65)*, *C50C3.8*, *epn-1*, *F01F1.6*, *F15C11.2*, *F23B12.5*, *T05F1.4*, *T23G11.7*, *tfg-1*, *Y11D7A.12*, *Y45F10D.13*, *F46H5.7* (*XH858*), *lin-10*, and *K04H4.2*. In addition, knock down of *dab-1* in *ehs-1* mutants resulted in severe phenotypes of *ehs-1*, such as larval lethality, molting and egg laying, to an extent that no worms were available for aldicarb experiments, indicating a genetic interaction between *ehs-1* and *dab-1*.

11.3.3 *rme-1* genetic interactions

Even though *rme-1* mutants did not demonstrate a phenotype related to aldicarb sensitivity, several genetic interactions were revealed, before the knock down experiments were carried out for its specific phenotype of reduced brood size. A total of six genes, which were identified as interactors by the other three EH proteins, and were not shown to interact with RME-1 by criss-cross transformation (Table 2), were found to interact with *rme-1* genetically. The RNAi of *dab-1*, *pcn-1* and *vab-19* caused the average "survival" time of *rme-1* mutants to be reduced more dramatically than what was observed in the RNA*i* of *C50C3.8*, *F46H5.7* (*XH858*), whereas the knock down of *Y11D7A.12* appeared to increase the average "survival" time (Figure 30a).

11.3.4 *reps-1* Genetic interaction

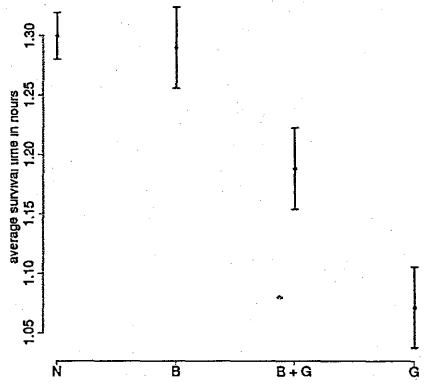
Reps-1 was found to interact with 10 genes out of 20 genes tested. It was observed that the time of "survival" decreased even more than *reps-1* on its own when the putative

interactors were knocked down. The ten genes that were found to interact genetically with *reps-1* are as follows: *B0041.2*, *BE003N10.3*, *epn-1*, *F15C11.2*, *K04H4.2*, *pqn-32*, *sel-5*, *T05E7.5*, *tfg-1*, and *vab-19* (Figure 31a).

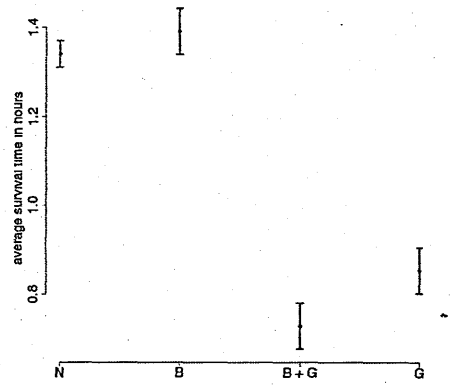
Figure 30. The average survival time under treatment with 0.5mM aldicarb of rme-1 mutant worms in which 26 genes were knocked down by RNAi: **N2 Bristol (N), N2 (RNAi) (G), rme-1 (B),** *and* **rme-1 (RNAi) (B+G).** *(n=40, in triplicates)*

a. *rme-1*: genetic interactions found

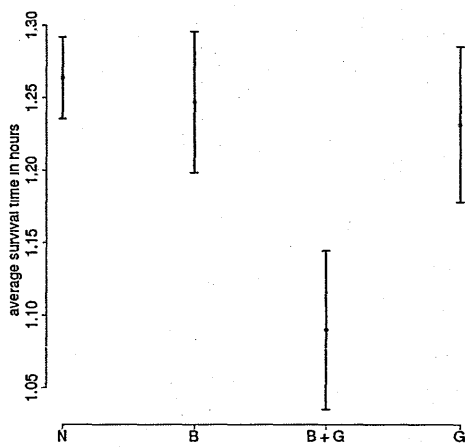
1) *rme-1 (RNAi;C50C3.8)*



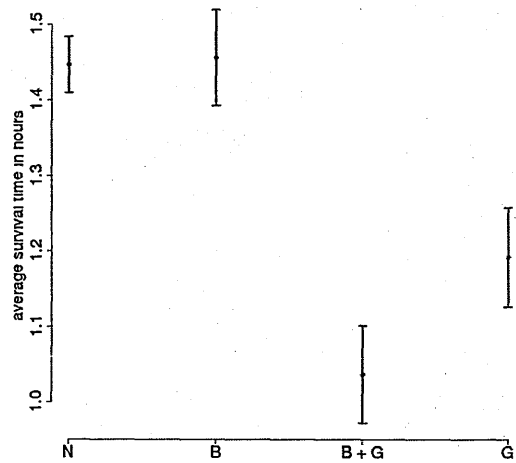
2) *rme-1 (RNAi;dah-1)*



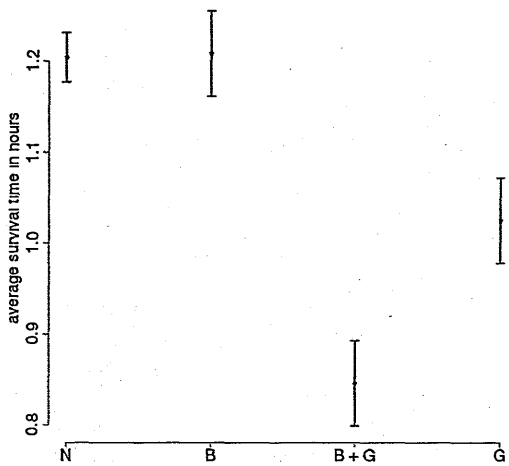
3) *rme-1 (RNAi;F46H5.7)*



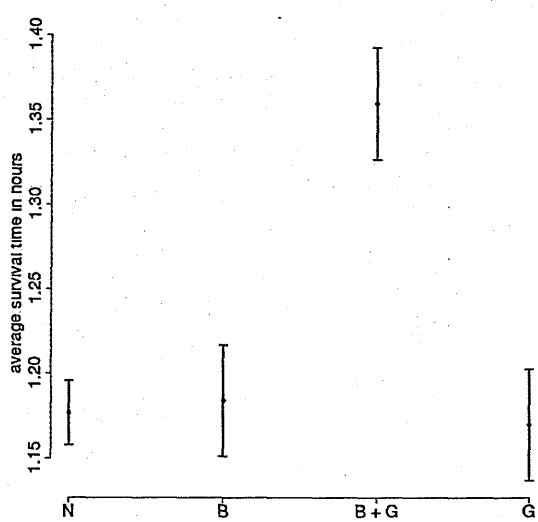
4) *rme-1 (RNAi;pcn-1)*



5) *rme-1 (RNAi;vab-19)*

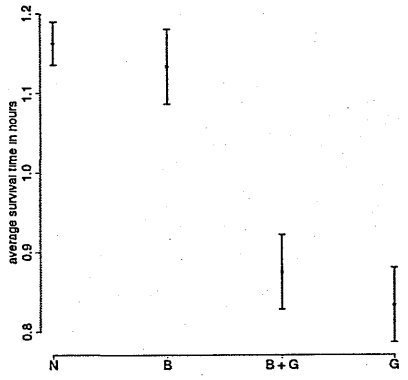


6) *rme-1 (RNAi;Y11D7A.12)*

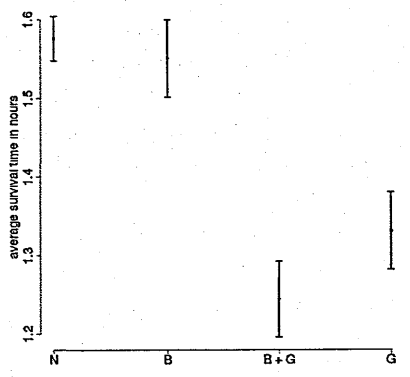


b. *rme-1*: No genetic interaction found

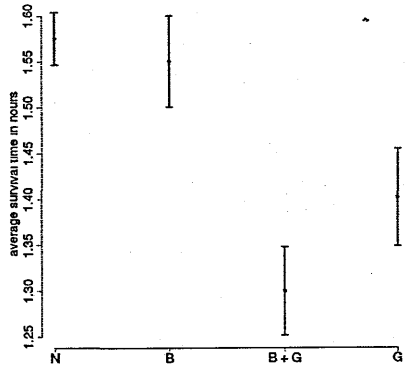
1) *rme-1 (RNAi;D1081.7)*



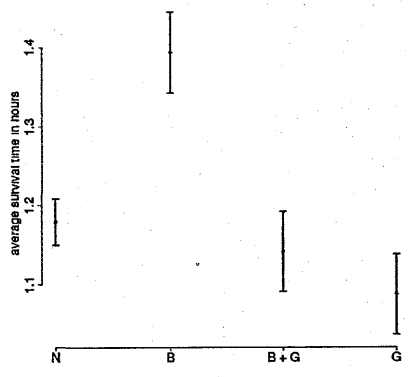
2) *rme-1 (RNAi;alx-1)*



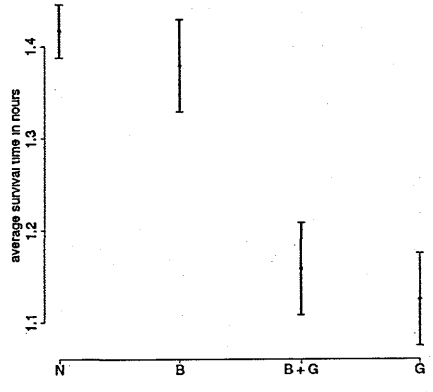
3) *rme-1 (RNAi;BE0003N10.3)*



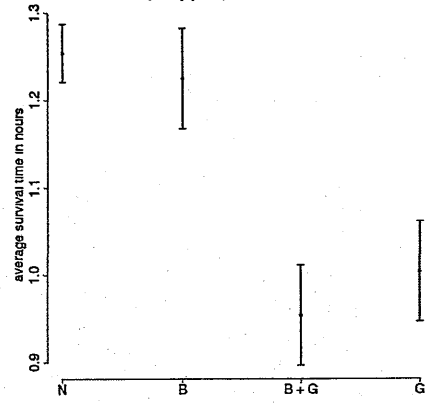
4) *rme-1 (RNAi;F01F1.6)*



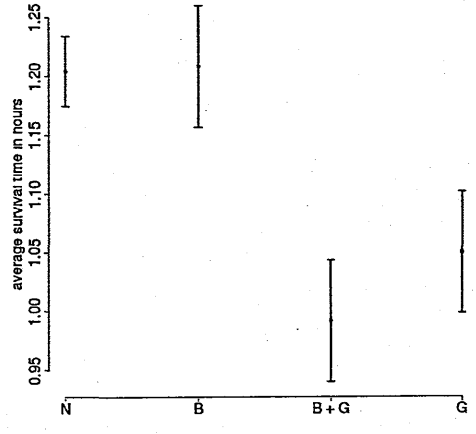
5) *rme-1 (RNAi;F23B12.5)*



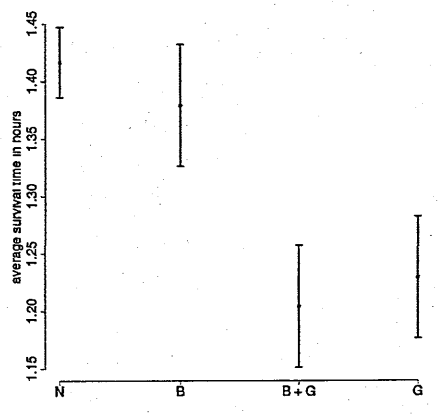
6) *rme-1 (RNAi;pqn-32)*



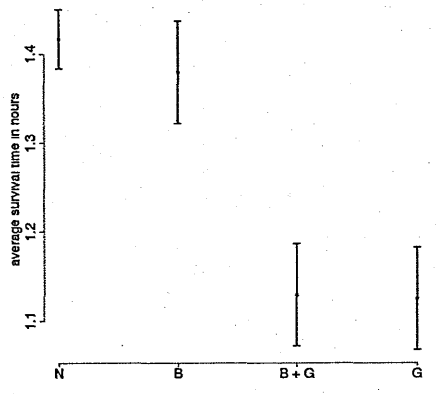
7) *rme-1 (RNAi;sel-5)*



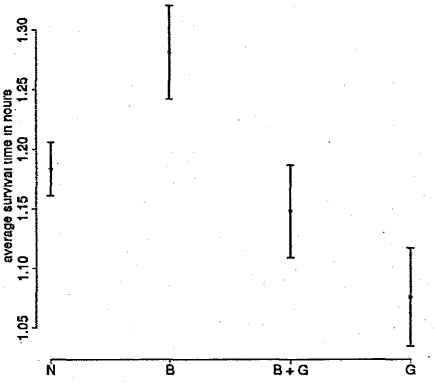
8) *rme-1 (RNAi;T05E7.5)*



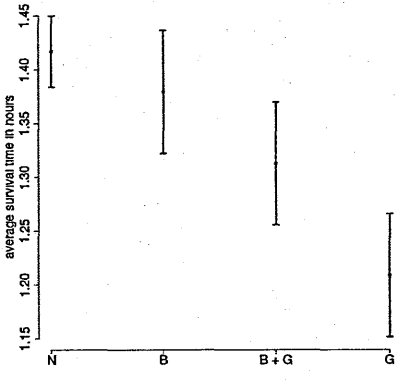
9) *rme-1 (RNAi;T05F1.4)*



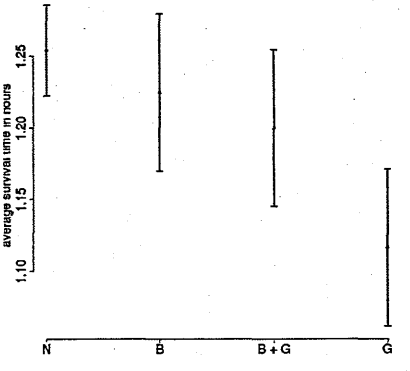
10) *rme-1 (RNAi;T23G11.7)*



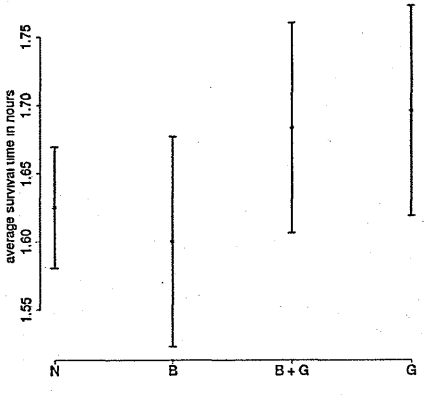
11) *rme-1 (RNAi;Y37E3.11)*



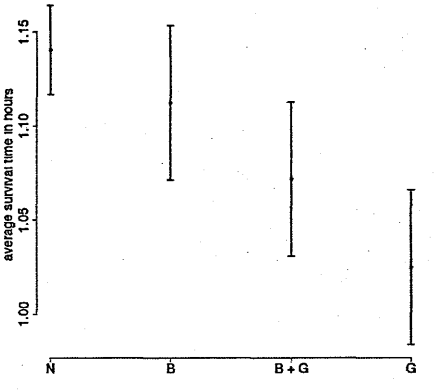
12) *rme-1 (RNAi;B0041.2)*



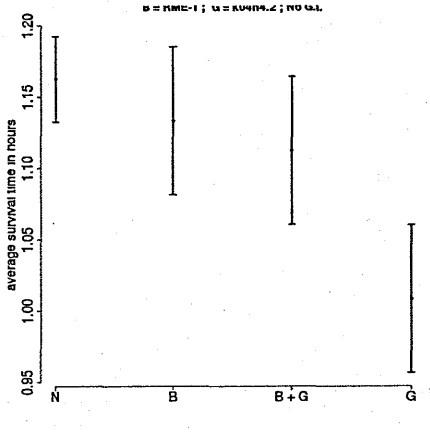
13) *rme-1 (RNAi;epn-1)*



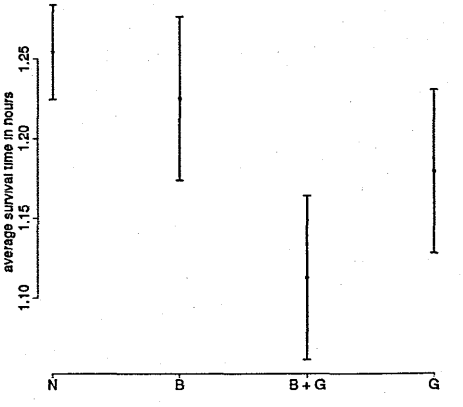
14) *rme-1 (RNAi;F15C11.2)*



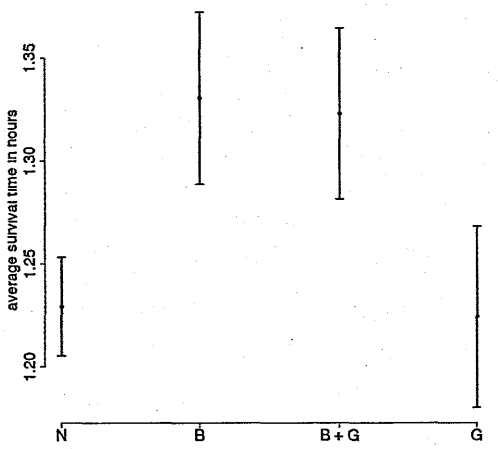
15) *rme-1 (RNAi;K04H4.2)*



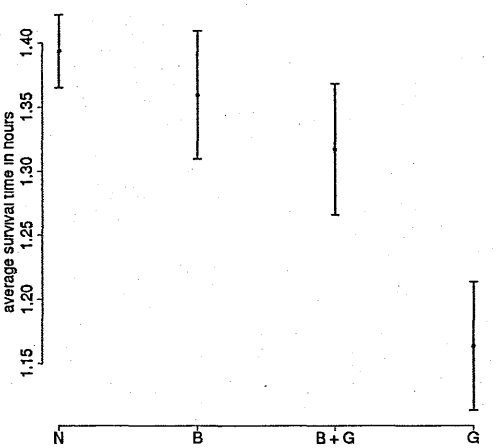
16) *rme-1 (RNAi;lin-10)*



17) *rme-1 (RNAi;M03A8.3)*



18) *rme-1 (RNAi;tfg-1)*



19) *rme-1 (Y45F10D.13)*

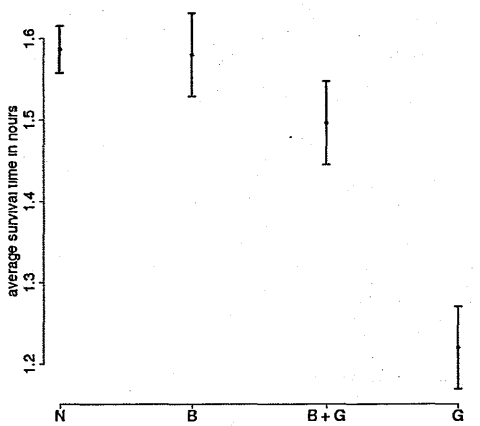
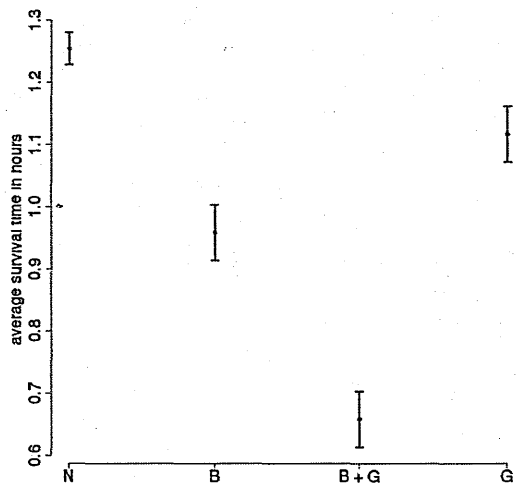


Figure 31. The average survival time under treatment with 0.5mM aldicarb of reps-1 mutant worms in which 26 genes were knocked-down by RNAi. N2 Bristol (N), N2 (RNAi) (G), reps-1 (B), and reps-1 (RNAi) (B+G).

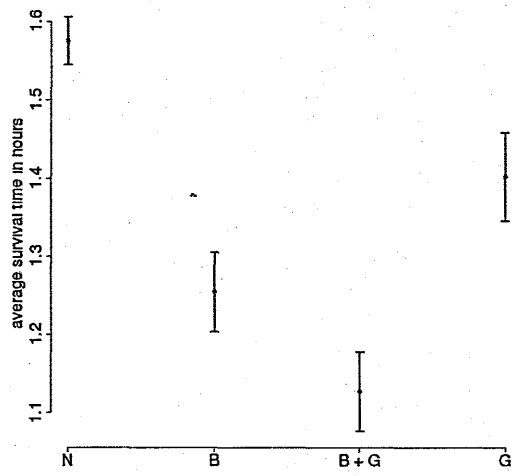
(n=40, in triplicates)
X-axis: The average survival time in hours under the treatment with 0.5mM aldicarb
Y-axis: N: wild type, B: itsn-1 mutant, B+G: itsn-1 mutant in which the specified gene was knocked down by RNAi, G: wild type in which the specified gene was knocked down by RNAi.

a. *reps-1*: genetic interactions found

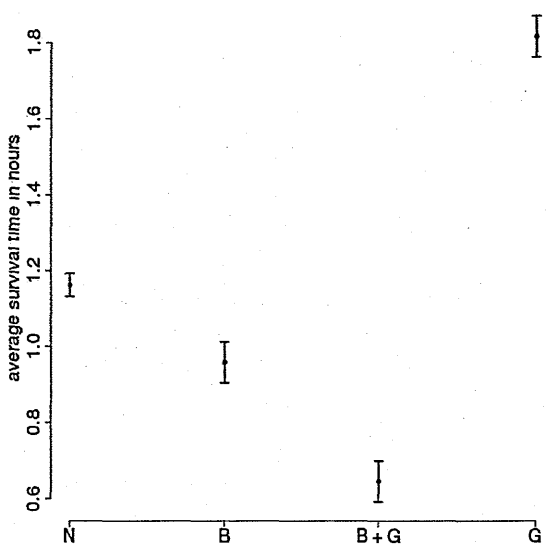
1) *reps-1 (RNAi;B0041.2)*



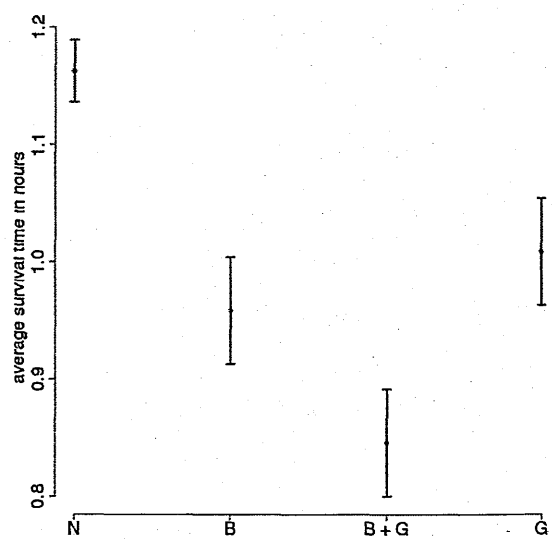
2) *reps-1 (RNAi;BE0003N10.3)*



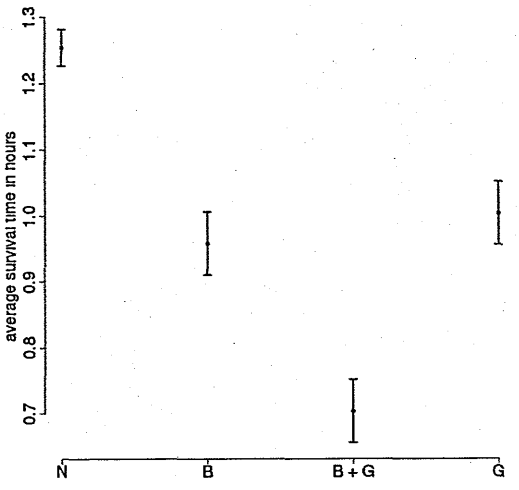
3) *reps1- (RNAi;epn-1)*



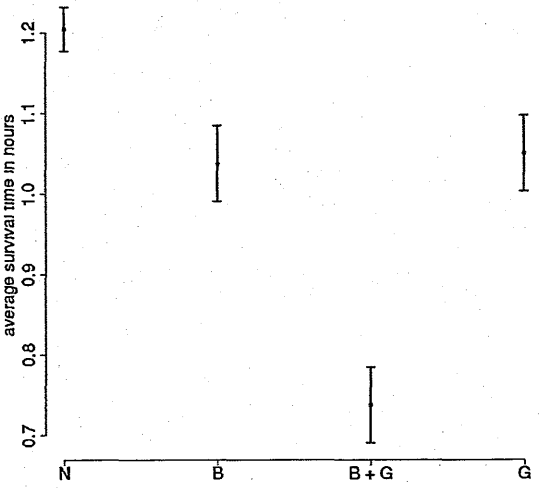
4) *reps-1 (RNAi;K04H4.2)*



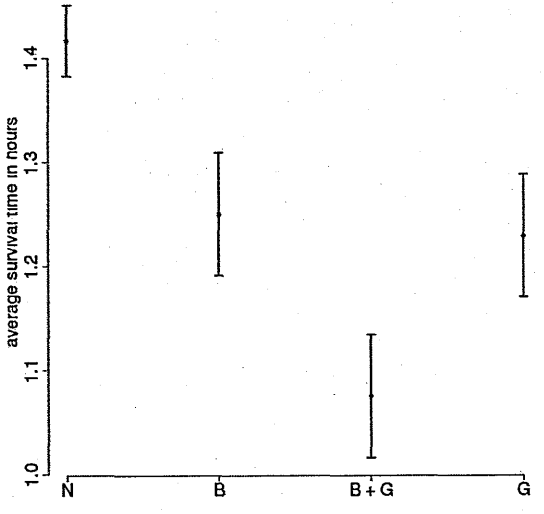
5) *reps-1 (RNAi;pqn-32)*



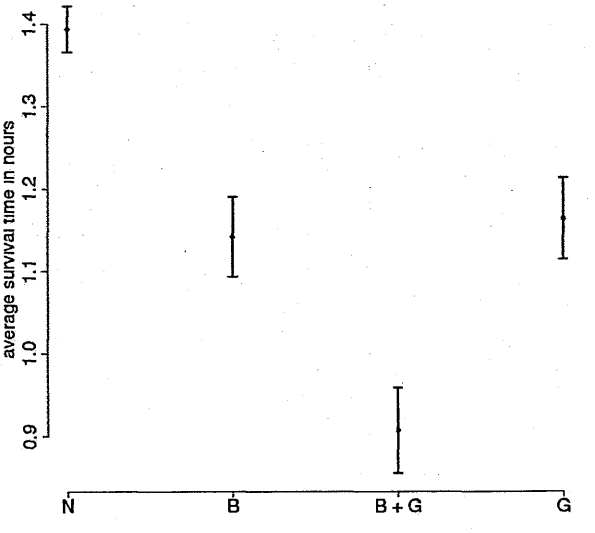
6) *reps-1 (RNAi;sel-5)*



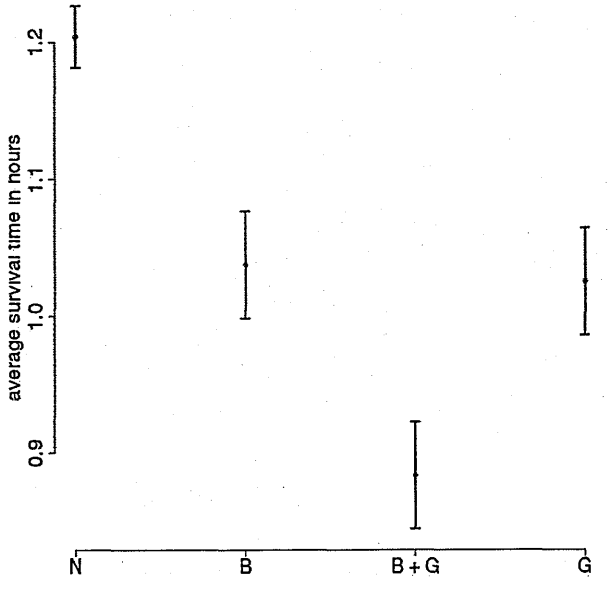
7) *reps-1 (RNAi;T05E7.5)*



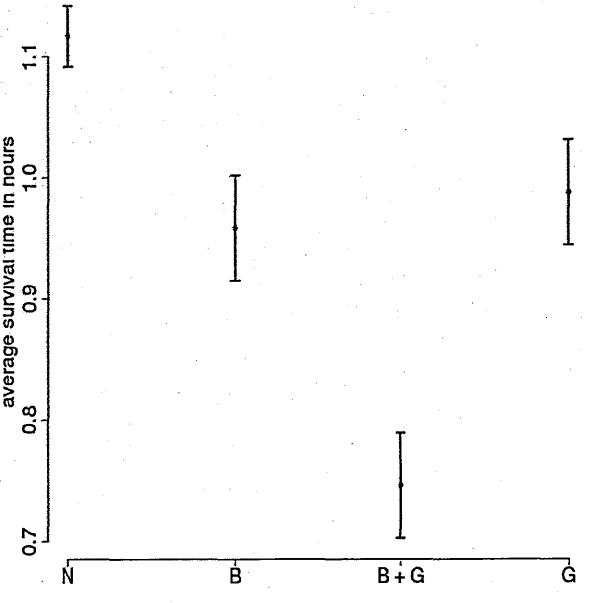
8) *reps-1 (RNAi;tfg-1)*



9) *reps-1 (RNAi;vab-19)*

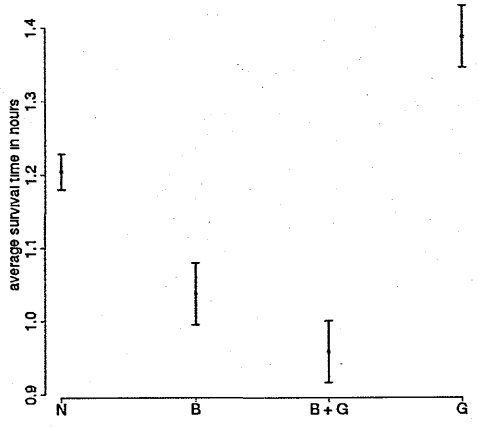


10) *reps-1 (RNAi;F15C11.2)*

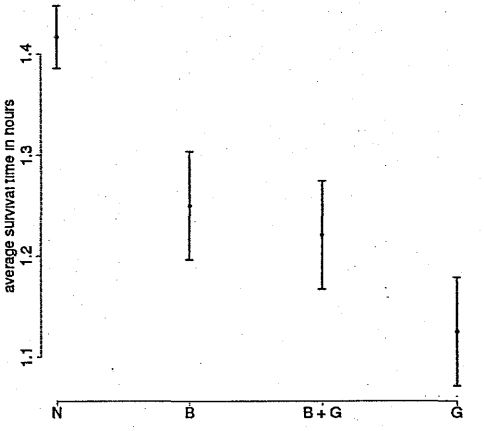


b. *reps-1*: No genetic interaction found

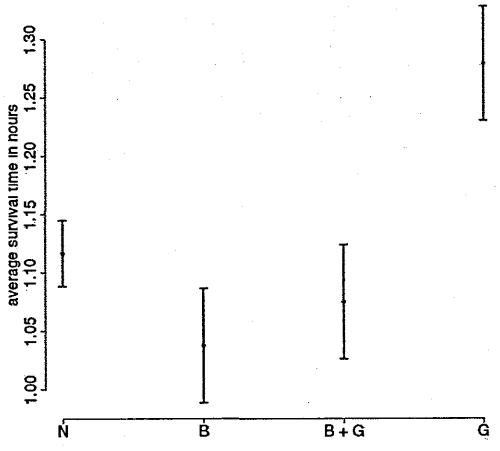
1) *reps-1 (RNAi;C50C3.8)*



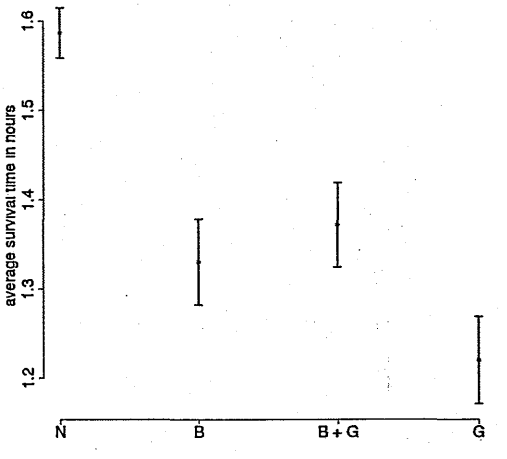
2) *reps-1 (RNAi;T05F1.4)*



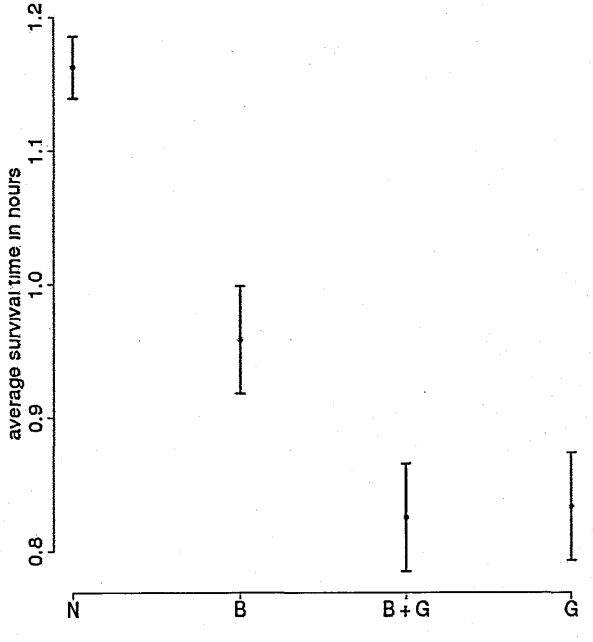
3) *reps-1 (RNAi;Y11D7A.12)*



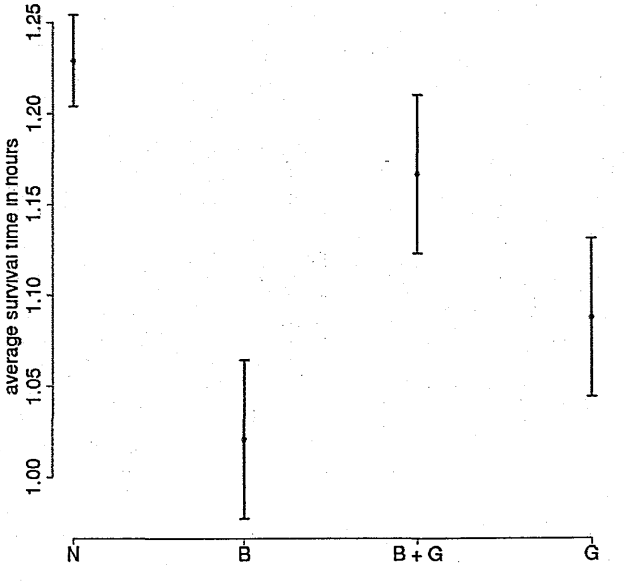
4) *reps-1 (RNAi;Y45F10D.13)*



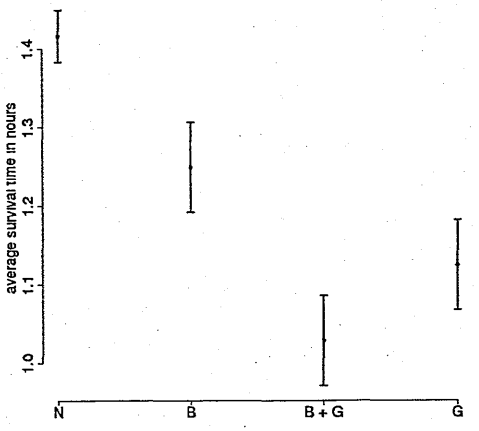
5) *reps-1 (RNAi;D1081.7)*



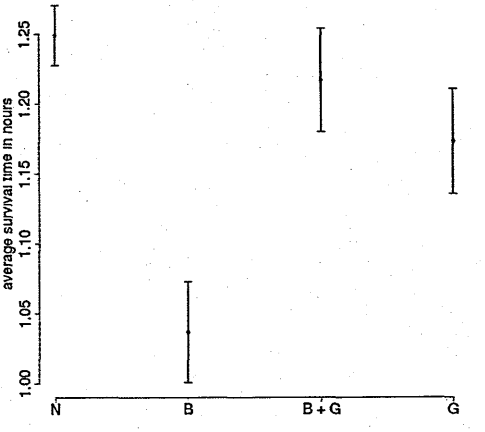
6) *reps-1 (RNAi;F01F1.6)*



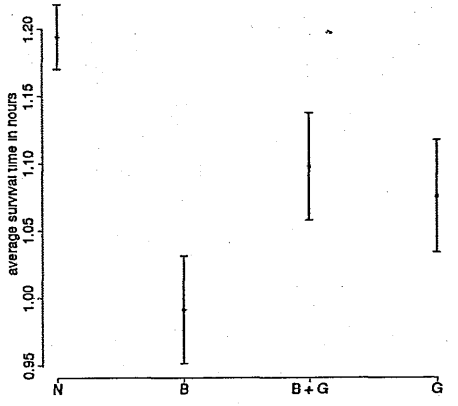
7) *reps-1 (RNAi;F23B12.5)*



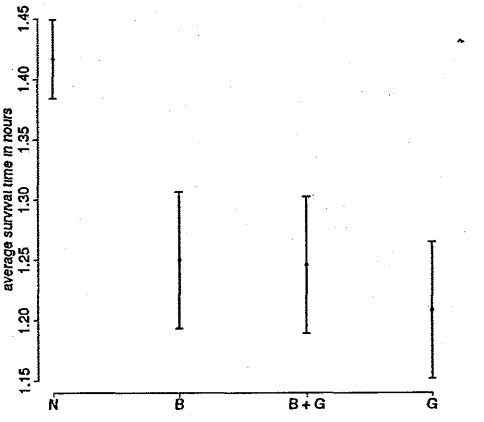
8) *reps-1 (RNAi;M03A8.3)*



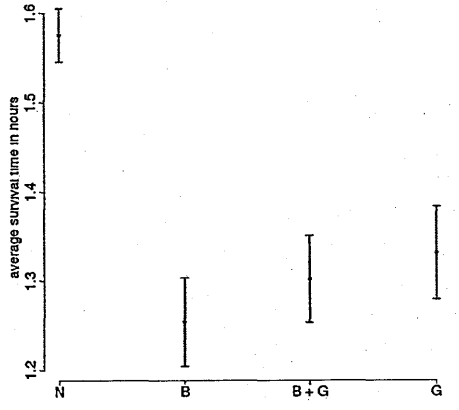
9) *reps-1 (RNAi;T23G11.7)*



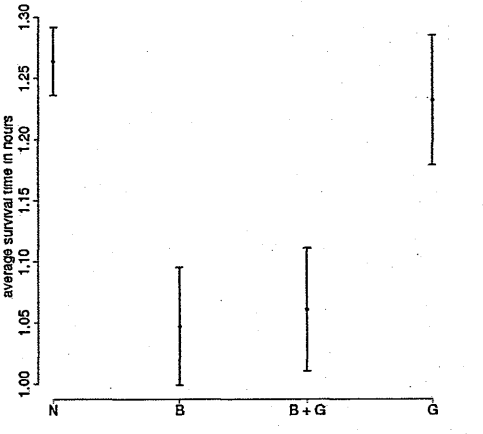
10) *reps-1 (RNAi;Y37E3.11)*



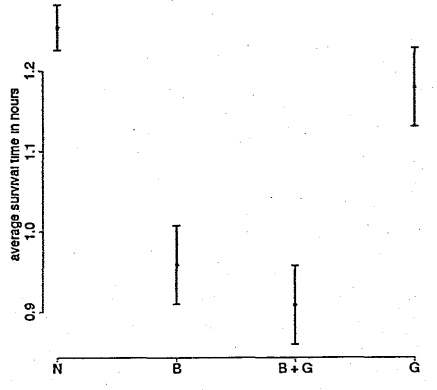
11) *reps-1 (RNAi;alx-1)*



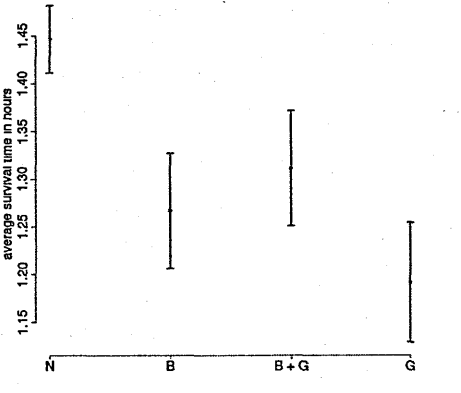
12) *reps-1 (RNAi; F46H5.7)*



13) *reps-1 (RNAi;lin-10)*



14) *reps-1 (RNAi;pcn-1)*



12. Genetic interaction 2: Brood size assay for RNA*i* in *rme-1* mutant strain

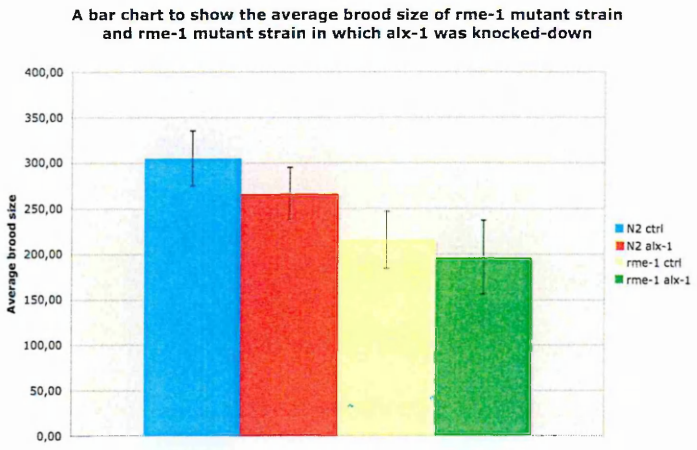
In order to test *rme-1* genetic interactions using a phenotype that is more appropriate for *rme-1* mutant (*b1045*), the effect of RNA*i* of the putative interactors on the brood size of *rme-1* was analyzed. Three putative interactors that were identified by criss-cross transformation were knocked down in *N2* and *rme-1* worms using RNA*i* by feeding. The change in the brood size apparently caused by the RNA*i* in *rme-1* was comparable to that observed in *N2* wild type worm. Thus we concluded that no genetic interactions were confirmed by monitoring the phenotype of decreased brood size of *rme-1*. The average brood sizes of the knock- down animals of ALX-1, Y37E3.11, and Y11D7A.12 in the genetic background of *N2* and *rme-1* are shown as bar charts in Figure 32. The brood sizes of 21 worms for each experiment used to calculate the average brood size are shown in Table 14.

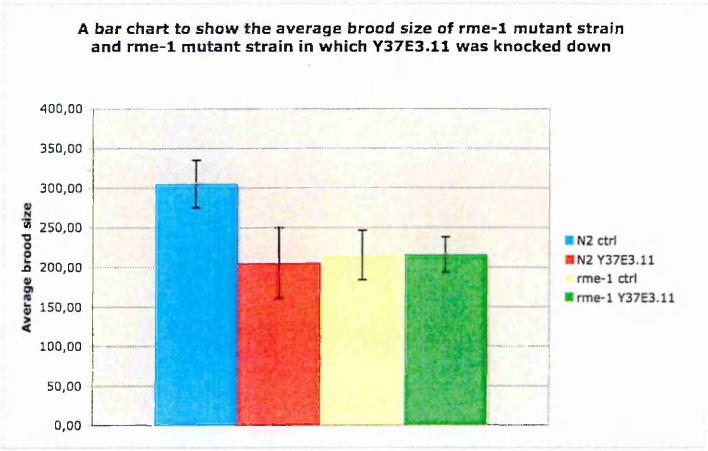
***Table 14 The average brood sizes of the* N2 *and* rme-1 *mutant worms in which* alx-1, Y37E3.11, *or* Y11D7A.12, *were knocked down by RNA*i.

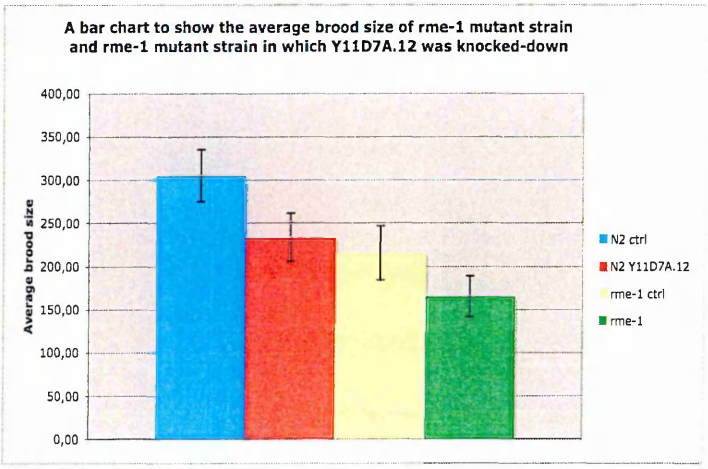
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Average	st dev
N2 ctrl	246	258	260	263	270	286	292	300	308	312	316	318	319	320	327	330	330	331	335	338	346	305.00	30.09
rme-1 ctrl	136	140	177	198	202	206	208	214	219	223	225	228	228	229	233	236	236	237	245	248	250	215.14	31.24
N2 alx-1	188	230	241	246	247	252	256	256	257	263	266	269	271	278	279	279	291	292	304	310	312	266.05	28.78
rme-1 alx-1	123	126	132	152	160	160	189	190	194	194	201	207	216	218	221	225	228	234	241	245	262	196.10	40.43
N2 ctrl	246	258	260	263	270	286	292	300	308	312	316	318	319	320	327	330	330	331	335	338	346	305.00	30.09
N2 Y37E3.11	109	143	151	162	168	170	176	180	194	202	210	216	226	230	239	242	244	252	258	267	267	205.05	44.71
rme-1 ctrl	136	140	177	198	202	206	208	214	219	223	225	228	228	229	233	236	236	237	245	248	250	215.14	31.24
rme-1 Y37E3.11	169	176	197	199	199	201	203	204	205	208	212	218	227	234	235	235	238	238	243	244	245	215.71	22.34
N2 ctrl	246	258	260	263	270	286	292	300	308	312	316	318	319	320	327	330	330	331	335	338	346	305.00	30.09
N2 Y11D7A.12	183	201	206	208	210	211	216	223	223	229	230	230	236	242	243	250	254	259	273	281	294	233.43	27.96
rme-1 ctrl	136	140	177	198	202	206	208	214	219	223	225	228	228	229	233	236	236	237	245	248	250	215.14	31.24
rme-1 Y11D7A.12	113	136	140	149	152	156	157	158	160	161	162	163	164	165	169	171	180	191	195	196	225	164.90	23.73

Figure 32. The average brood sizes of 21 **rme-1** *mutants and* **rme-1** *mutants in which* **alx-1**, **Y37E3.11**, *or* **Y11D7A.12** *were knocked down.*

The effect of RNAi of these genes in the wild type background of *N2 Bristol* strain is shown for each experiment.







13. Summary of interactions identified and the construction of the Interactome map

13.1 ITSN-1 interactors

13 proteins out of 26 interact with ITSN-1 physically in the Y2H system, of which 10 were validated by the *in vitro* binding assays. Probably for the fact that a full-length EH proteins were used in the *in vitro* binding experiments, some proteins that were identified to interact with one EH protein in the Y2H system were also found to interact with the other EH proteins in the *in vitro* pulldown assays. For example, Y11D7A.12, which was thought to interact only with EHS-1 and RME-1 by the Y2H system, was co-purified with the full-length ITSN-1.

13.2 EHS-1 interactors

EHS-1 was found to interact with 16 proteins by the Y2H screenings, of which 13 were confirmed by the *in vitro* pull-down assays. As observed in the pull-down assays for ITSN-1, two proteins, T05F1.4 and SEL-5, which were identified as a specific interactor of REPS-1 by the Y2H screenings in this study, were shown to interact also with EHS-1 by the pull-down assays. A genetic interaction was shown between *T05F1.4* and *ehs-1*, in addition to confirming the genetic interactions for 11 out of 16 physical interactors of EHS-1. The other five interactors that were found not to interact with *ehs-1* genetically could be explained by the fact that the protein interactions are not involved in the pathway that results in the phenotype detectable by the aldicarb sensitivity assay.

13.3 RME-1 interactors

The fact that the number of interactors found by RME-1 screening is so small may be due to the fact that the EH domain of RME-1 requires other domains in the protein. For example, it has been reported that ATP-binding to the p-loop located in the N-terminus

of the protein plays a role in oligomerization, which is suggested to be important for the functions of RME-1, and it may also promote interactions with other proteins, although the report does not investigate the interactions mediated solely by its EH domain [121]. An attempt to use a bait containing the p-loop together with the EH domain was not carried out because it would be a full-length bait. Such bait would include the coiled-coil region, located in the middle of the protein, and hence interfere with the aim to specifically identify EH-mediated interactors. Indeed, the full-length RME-1 expressed as a GST-fusion protein in the *in vitro* pull-down assays detected an array of interactors that were shown by Y2H system to interact only with the EH domains of ITSN-1, EHS-1, and REPS-1, such as EPN-1, DAB-1, and LIN-10.

13.4 REPS-1 interactors

The Y2H screenings of REPS-1 identified 17 proteins of which 8 were confirmed to interact genetically. In addition, 2 proteins that were shown to interact with ITSN-1 and EHS-1 by Y2H system and 1 protein which had been identified as an interactor specific to ITSN-1 was shown to interact genetically with REPS-1. Unfortunately the *in vitro* pull-down assays of REPS-1 did not identify any GST-fusion proteins of the putative interactors.

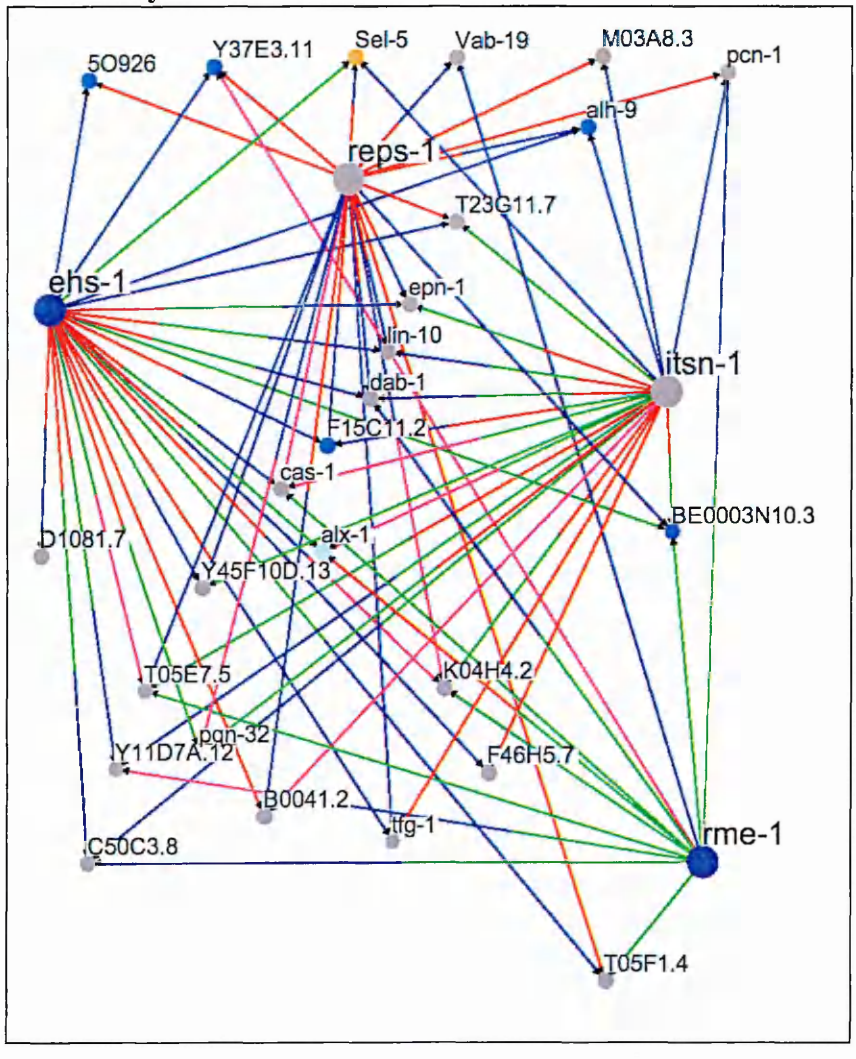
13.5 The EH Interactome map

Innteractome maps that represent the putative interactions found by Y2H screenings, *in vitro* pull-down assays and by genetic analysis were created using the software Osprey 1.2.0, and is shown in Figure 33. The interactions found by the three different approaches are also summarized in Table 12. The putative mammalian homologues of the interactors identified, where known or predictable, will be discussed later in Section 16 (See Table 14).

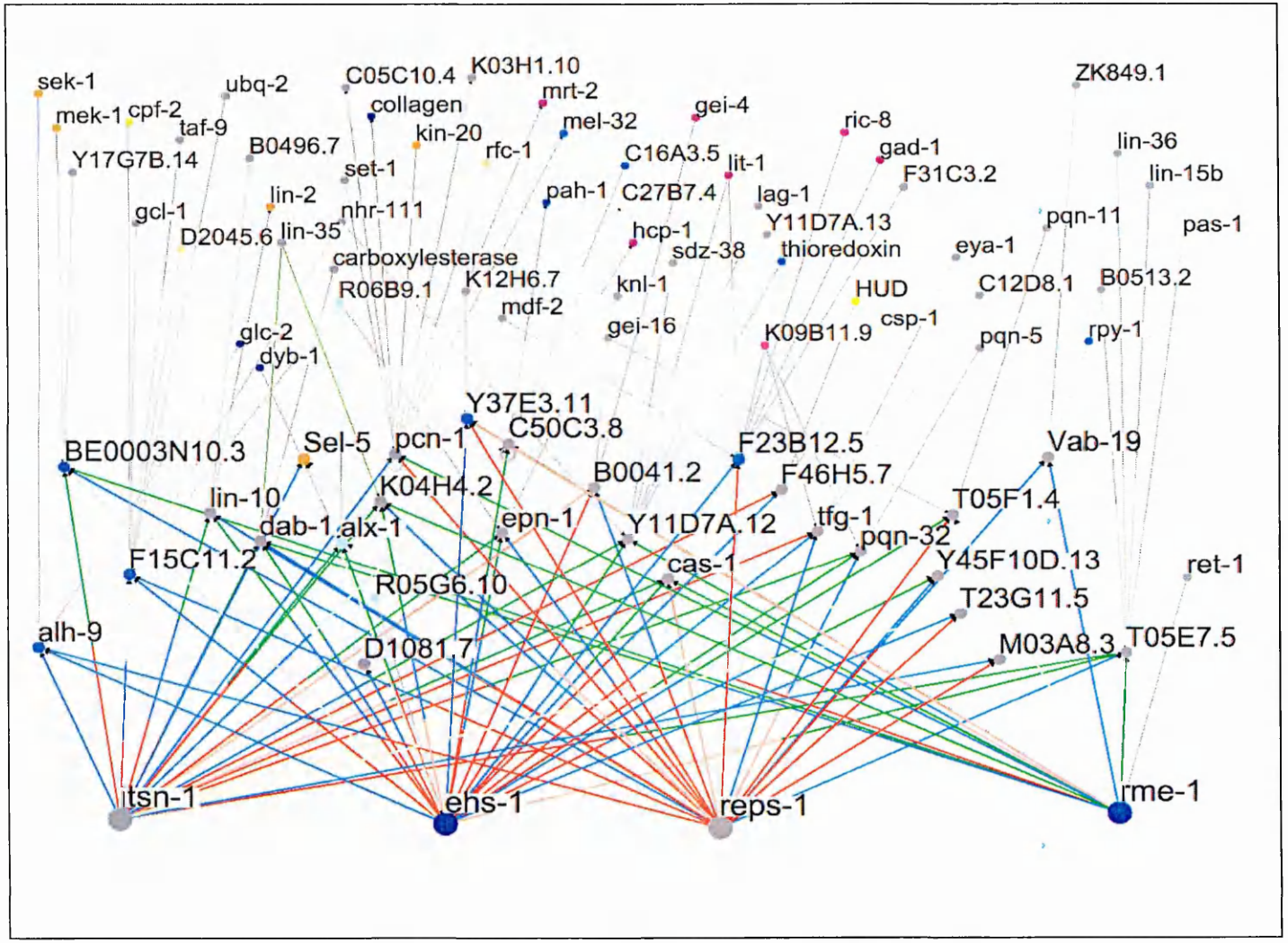
Figure 33 Map of interaction of EH domain-containing proteins identified by Y2H screening, criss-cross re-transformation, In-vitro binding, and genetic interaction, generated using the software Osprey _1.2.0.

Four EH proteins, ITSN-1, EHS-1, REPS-1 and RME-1 are labelled with the largest cirles, in the interactome map. The interactors of the EH proteins identified in this study are shownwith the medium-sized circles in the map, right above the bottom layer. The methods by which the interactions were identified in this study are indicated by different colours of the lines; red lines: Y2H screening; pink lines: Criss-cross re-transformation; green lines: *In-vitro* binding; and blue lines: genetic interactions. Where there are more than one colour per interaction, the colours indicate different methods by which the interactions were confirmed. The proteins notated with smaller circles and the interactions notated in grey are the known interactors of the interactors found in this project, or of the EH proteins [138].
The circles representing each protein are colour-coded automatically by the Osprey 1.2.0, to represent the general function of the proteins, where known, as follows: Purple: Transport, Blue: Metabolism, Light blue: Signal Transduction, Brown: Protein phosphorylation, Bright pink: Protein transport, Beige: Protein degradation, Green: RNA processing, Dark beige: DNA repair, Light pink, DNA damage response, Violet: Cell organization and Biogenesis. Note that these annotations were applied automatically in the function within the Osprey 1.2.0, based on the information available from the Gene Ontology (GO) Consortium.
The mammalian homologues of the EH interactors, where known, are summarized later, in Section 16 and in Table 14.

a. An Interactome map to show the four EH proteins and their interactors identified in this study.



b. An interactome map of EH proteins in *C. elegans*. The map shows the the four EH proteins and their interactors identified in this study, and the reported interactors of the EH interactors in *C. elegans*, as reported in the Wormbase interaction database. (http://www.wormbase.org).
The EH proteins are shown at the bottom of the map, each labelled with the largest circles. The interactors of the EH proteins are clustered in the middle section of the map labelled with the middle-sized circles, whereas their interactors are labelled with the smallest circles and the lines representing the interactions in grey. The colour coding of the map is as described in the beginning of Figure 33.



IV. DISCUSSION

This project identified a total of 26 interactors by the Y2H approach, of which 17 were validated to bind to at least one of the EH proteins in *C. elegans* by either *in-vitro* pulldown or assays for monitoring genetic interactions. It was the first approach to identify the interactors of EH proteins on a genome-wide scale. It appears clear, from an overall look at the list of the EH interactors identified during this project, as demonstrated in the interactome map in Figure 33 (and in Table 15 in the later section) that many of the interactors are found in various intracellular events, holding together a network of adaptor proteins involved in all the major processes of the protein sorting events in intracellular signalling.

Our results implicate EH proteins and their interactors in sorting events such as receptor maturation, secretion, receptor internalization, multivesicular endosome (MVE) and multivesicular bodies (MVB) sorting, vesicle recycling, nuclear-cytosolic shuttling, protein degradation. Furthermore, the results from this study indicate that the EH network is involved in various signalling pathways at a more global level.

In the first part of the discussion, the previous knowledge on EH interactors in *C. elegans* will be addressed in the light of what was found in this study. In the second part, the future experiments that will help to answer the questions on *C. elegans* EH proteins raised in this study will be proposed. The final part of the discussion will look at some of the EH interactors identified in this study, or their homologues in other species, to build a speculative model on the role of EH network in intracellular

signalling, and diseases such as cancer, human immunodeficiency virus-1 (HIV-1) infection and neurodegenerative diseases.

14. Previously known and predicted EH interactors in *C. elegans* confirmed by this study

14.1 Previously known EH interactors in *C. elegans* confirmed by this study

Apart from a few interactions that had already been demonstrated previously by others, mostly in mammalian systems, most of the proteins identified as putative interactors of *C. elegans* EH proteins during this project are novel. Two EH-mediated interactions discovered in this study have been reported for *C. elegans* EH proteins, namely the interaction between *alx-1* and *rme-1* [101] and the interaction between *ehs-1/itsn-1* with *dab-1 [139]*, supporting the validity of our screening. The interaction between *alx-1*, or *dab-1*, with the other EH proteins has not been reported to date.

14.2 Predicted EH interactors confirmed by this study

Two *C. elegans* proteins had been previously predicted (but not demonstrated) to interact with EH proteins and both were identified in this project [18]. LIN-10 is part of a trimeric complex LIN-2/LIN-7/LIN-10, which plays a role in the basolateral localization of EGFR LET-23 in vulval epithelial cells [140]. In addition, its mammalian homologue Mint-1 (Munc18 interacting protein 1) is essential for synaptic vesicle exocytosis [141, 142]. LIN-10 was a possible candidate for an EH interactor as it contains 2 NPF motifs and its functions are related to trafficking [18]. The prediction of LIN-10 as an EH interactor was confirmed in this project, as it was demonstrated to interact with ITSN-1, EHS-1 and REPS-1 by the Y2H screenings.

The other predicted EH-binding protein SEL-5, was also identified in this study as an interactor of REPS-1. SEL-5 also contains one NPF motif as well as a class II EH-interacting motif, HSF. SEL-5 is a serine-threonine kinase that facilitates the LIN-12/Notch receptor activity [143, 144]. Notch in *Drosophila* had been shown to require an endocytic protein dynamin for its function [145, 146]. The interaction between REPS-1 and SEL-5 further supports a role for endocytosis and the EH proteins in the Notch signalling pathway. Conversely, Numb, a Notch antagonist, is able to interact with Eps15 through its NPF motif [11, 147], even though *C. elegans* Numb was not identified in our screenings. Indeed, the *C. elegans* homologue of Numb, num-1 does not contain any EH interacting motif, proving the specificity of the screening.

In addition, the mammalian homologues of two interactors identified in this study have already been described as EH interactors. Epsin is known to interact with Eps15 [13], Intersectin [14] and Reps1 [148]. Ubiquilin (hPLIC), the predicted human homologue of F15C11.2, has been shown to interact with Eps15. The EH domains are not essential for binding between mammalian Eps15 and Ubiquilin [149]. However, this study has indicated that, in *C. elegans*, the interactions between F15C11.2 and different EH proteins is mediated by the EH domain: the baits designed for EHS-1 and ITSN-1 contained only restricted regions of EH domains, yet both baits were able to interact with F15C11.2. Moreover, the EH-mediated interaction with F15C11.2 is likely to be facilitated by the regions surrounding the EH domain, since the REPS-1 bait that contained an extended region around the EH domain used in this study demonstrated a stronger interaction with F15C11.2 than EHS-1 or ITSN-1.

15. Further experiments to be done on *C. elegans* EH proteins

15.1 Is R10E11.6 an EH protein ?

R10E11.6 is a gene that was recently identified as an EH-domain containing protein. The gene is uncharacterized, and despite its sequence similarity to the mammalian γ-synergin family, as discussed in Section 11.5, the predicted EH domain of the *C. elegans* homologue did not show any interaction with NPF-containing proteins. The NPF containing proteins tested were those that were identified as the interactors of the other three EH proteins in this study, in addition to SCM-1, which was predicted to interact with R10E11.6, as discussed in Section 11.5.3. The lack of a mutant worm for R10E11.6 renders an appropriate analysis of its function in nematode difficult. However, R10E11.6 is in an operon with *vha-1* and *vha-2*, encoding subunits of the vacuolar proton-translocating ATPase, suggesting that it might be required for lysosomal biogenesis.

To date, this study was unable to verify whether R10E11.6 is a true EH domain-containing protein. This study investigated whether the EH domain of R10E11.6 was able to interact with any of the interactors of the other *C. elegans* EH proteins, and no interaction was observed. Furthermore, the EH domain of R10E11.6 did not show interaction with SCM-1, a homologue of an interactor of γ-Synergin in a re-transformation assay in this study.

The EH domain of R10E11.6 did not interact with any of the *C. elegans* NPF-containing proteins that bind to the EH domains of the other proteins. In addition, to the co-transformants of the bait of R10E11.6 with the prey containing a putative interactor SCM-1 did not show any activation of the reporter gene *LacZ*. These observations suggest that R10E11.6 may not be an EH protein, and therefore it may not be a true homologue of γ-synergin. Functional characterization of R10E11.6 mutant, once

available, will enable us to determine whether R10E11.6 acts as a homologue of γ-Synergin. In addition, interactions with other proteins that bind to γ-Synergin, mediated by regions outside of the EH domain, such as Aftiphilin and p200 [129], will also help to answer whether R10E11.6 is a homologue of γ-Synergin. Moreover, the region recognized as the EH domain of R10E11.6 can be tested by *in vitro* binding assay with GST-fusion peptides containing NPF motifs, which was shown in a previous study to interact with the EH domain of Eps15 [11]. However, the GST-NPF-containing peptides were taken from the sequences of the mammalian proteins, Hrb/Hrb-l and Numb/Numb-l, and if the EH domain was unable to interact with the *C. elegans* NPF-containing proteins in this study, it might not interact with the mammalian NPF-containing peptides.

15.2 Interactions still to be confirmed by *in vitro* pull-down assays

8 proteins out of a total of 26 identified in our screenings were not cloned in-frame as GST-fusion proteins. These are marked as "N/A" in the column for the pull-down experiments in Table 12. Therefore the pull-down assays in this study were considered as validations only for the interactions between the EH proteins and the 18 proteins cloned; most of these may be mediated by a NPF/DPF motif. Validation by *in-vitro* binding assay of an EHS-1 specific interactor, D1081.7, which contains a class II EH-interacting motif (HTF) and a class III EH-interacting motif (FWR), will help to confirm the interactions mediated by these other classes of interacting motifs. In this context, it is worth noting that Y45F10D.13, an HSF-containing protein, was validated as an interactor of EHS-1. Y11D7A.12, which contains an FWR motif, was also confirmed to interact with ITSN-1, EHS-1 and RME-1 by *in vitro* binding assays.

15.3 REPS-1 specific interactions

Of the eight proteins that were not validated by *in vitro* pull-down assays, five were REPS-1 specific interactors (two of the seven REPS-1 interactors were validated). However, it must be pointed out that none of the *in vitro* pull-down assays for REPS-1 showed binding to any putative interactors, in spite of the fact that the FLAG-tagged REPS-1 did interact with a GST-fusion peptide containing the NPF motif (which suggests that the EH domain in the fusion protein was folded correctly enough to recognize the peptide motif). The fact that no GST-fusion proteins larger than the peptide motif were shown to interact with REPS-1 could lead to two speculations. One is that the interactions identified by the EH domain of REPS-1 in Y2H screenings are artefacts of Y2H system. However, this does not explain the numerous genetic interactions observed in the mutant background of REPS-1 that validate the Y2H interactions identified in this study. The second is that the folding of the FLAG-tagged REPS-1 was not efficient enough to recognise the binding region in the GST-fusion contest. Since the sequence of REPS-1 present in the Wormbase was not constant during the period of this project, FLAG-tagged REPS-1 was expressed as the same fragment used as the bait REPS-1 in the Y2H screenings, as the exon coding for the EH domain was found constant in all sequences of REPS-1 found to date. The fact that a fragment of REPS-1 was expressed in a heterologous system, could have prevented the correct conformation of FLAG-REPS-1 and the binding, even though the bait was functional in another heterologous system, that of yeast in the Y2H.

The analysis of *reps-1* gene is further complicated by the lack of ESTs available, suggesting three possibilities about REPS-1 protein expression in *C. elegans*: 1) the expression is at a very low level, 2) the expression is in few specific cells or 3) the expression is limited within a specific time frame during development. Further experiments, such as studying the expression pattern of REPS-1 in *C. elegans* by using

GFP-fusion proteins of REPS-1 expressed under the *reps-1* promoter, will indicate when and where REPS-1 is expressed in the nematode, thereby facilitating isolation of the ESTs of REPS-1, and cloning the full-length endogenous REPS-1 to elucidate its currently ambiguous sequence.

In the Y2H screenings, the REPS-1 bait contained regions outside of the EH domain. Seven interactors that do not contain NPF or other known EH-interacting motifs were identified in REPS-1 Y2H screenings. The extra region surrounding the EH domain is likely to be responsible for binding with these REPS-1 specific interactors. This is also supported by the fact that none of the other baits used (from the other EH-containing proteins screened) were able to interact with these REPS-1 specific interactors when criss-cross re-transformations were performed. A bait containing a mutated EH domain of REPS-1 should clarify whether these REPS-1 specific interactions are EH-mediated. No common motifs were identified in the sequences of these 7 REPS-1 specific interactors in this study, but if the interaction is indeed mediated by the EH domain, then a closer analysis of the sequences to identify new EH-interacting motifs is warranted.

15.4 RME-1 interactors

A number of recent findings on the EHD family of proteins (of which RME-1 is a member) indicated that nucleotide-binding to the EHD proteins is required for the interactions mediated by its EH domain (Refer to the sections 2.5.1 and 2.5.4). In addition, oligomerization of the EHD proteins, which also requires the nucleotide-binding, is thought to be critical for the EH interactions.

The Y2H screenings of RME-1 in this study was carried out using the C-terminal EH domain alone as the bait. This means that the bait lacked both the oligomerization domain located in the coiled-coil region in the central portion and the nucleotide-binding domain in the N-terminal of the protein. Full-length proteins were not used in the Y2H screenings in this study to avoid the interactions mediated by non-EH regions. Indeed, a strong interaction was observed in the X-gal assay between ALX-1 and RME-1 only when ALX-1 was co-transformed with the bait J, which contains the coiled-coil region. It is probable that the bait J was able to oligomerize to some extent even without nucleotide-binding, and therefore assisted the interaction with ALX-1, in comparison to other baits such as bait D and F. Despite the risk of identifying non-EH interactors, use of the full-length bait to perform an Y2H screenings, should therefore reveal further novel interactors of the EH domain of RME-1.

16. Roles of the extensive intracellular EH network based on the EH interactors identified

The EH network that has been uncovered in this study appears to span diverse intracellular compartments from which different signalling pathways are conducted. Although many of the *C. elegans* EH interactors identified in the screenings are uncharacterized or do not have clear homologues in other species, the functions of protein families such as the Epsin family, Disabled family, Lin-10/X11/Mint1 family, Alix family, and SEL-5/GAK1 family have been reported. The *C. elegans* homologues of these families, EPN-1, DAB-1, LIN-10, ALX-1 and SEL-5 were identified as interactors of more than one EH protein in this study. By looking at the previously known functions of these protein families, the extent of the EH network can be visualized, and help to speculate on the intracellular role of the EH network.

Furthermore the screening identified novel interactors, adding complexity to the EH-network.

The range of proteins identified to interact with EH proteins in this study show that the EH network contains several signalling pathways such as EGFR/Ras/MAPK pathway, Notch/LIN-12 pathway, and Hedgehog signalling pathway. The adaptor proteins Epsin, LIN-10, and CAS-1/CAP that interact with EH proteins and participate in the endocytic protein sorting or actin cytoskeleton organization in the EGFR/Ras/MAPK signalling pathway will be discussed. In addition, several components of Notch/LIN-12 pathway have been identified as EH interactors, such as SEL-5, EPN-1, and ALX-1. The interaction identified in this study between EHS-1 with C50C3.8, which is most likely to be a homologue of *Drosophila* roadkill, is the first example of an EH protein being implicated in Hedgehog signalling.

In the following section, the EH interactors identified in this study in *C. elegans*, of their homologues in other organisms will be discussed, divided into sub-sections according to their functions in the intracellular trafficking. The *C. elegans* proteins and their homologues that will be discussed in the following sections are shown in Table 15 below.

Table 15 The EH interactors and their homologues that have been implicated in various intracellular processes.

	Protein Names			
Intracellular processes	***C.elegans***	***H. Sapiens***	***S. cerevisiae***	***D.melanogaster***
Endocytosis	EPN-1 [**see below]	Epsins [13]	Ent1p/Ent2p [150]	Iqf
	F15C11.2	Ubiquilins (PLIC-1/-2) [149, 151]	Dsk2p	CG14224
	ALX-1 [101]	Alix [152]	Bro1p	ALiX
	DAB-1	Dab1/Dab2 [154-156]	-	Dab
Actin cytoskeleton dynamics	CAS-1	Cap1 [158]	Srv2p [157]	CG33979
	F15C11.2	Ubiquilins (PLIC-1/-2) [159,160]	Dsk2p	CG14224
Receptor maturation	LIN-10 [140,161-163]	Mint1	Muc1p	X11Lbeta
Ligand sorting	EPN-1	Epsins	Ent1p	Iqf [164,165]
Ligand secretion	DAB-1 [167]	Dab1/Dab2	-	Dab
	TFG-1	(COPII complex?) [170]	Vrp1p	CG7709
Early endosome trafficking	SEL-5 [144]	GAK1 [173]	Ark1p/Prk1p (Pak1p) [171]	Nak
MVE/MVB sorting	ALX-1 [174]	Alix [175,176]	Bro1p	ALiX
Receptor recycling	ALX-1 [101]	Alix	Bro1p	ALiX
Nuclear functions	EPN-1	Epsins [181]	Ent1p	Iqf
	C50C3.8	Speckle-type POZ protein	-	Rdx [182]
Protein degradation	F15C11.2	Ubiquilins (PLIC-1/-2)	Dsk2p [184]	CG14224
	BE0003N10.3	FBX11?	-	FBX11?

[**] Personal communication from Dr. MG Malabarba

16.1 EH interactors in Endocytosis

The canonical function of EH domain-containing proteins to date is the endocytic function. Proteins that belong to Eps15 family, Intersectin family, EHD family and Reps/POB family have each been shown to participate in endocytosis or in endocytic recycling processes. Mammalian Eps15 and Epsin were shown to interact via an EH-NPF interaction, and their role in endocytosis has been demonstrated [13]. In this study, *C. elegans* Epsin homologue EPN-1 was shown to interact with EHS-1, *C. elegans* Eps15, as well as with ITSN-1 and REPS-1. The yeast epsins, Ent1p and Ent2p are also essential for endocytosis, particularly the epsin N-terminal homology (ENTH) domain, found conserved in Epsin homologues in *C. elegans* to mammals. In addition, the C-terminal region of Ent1p was shown to bind clathrin [150]. The *C. elegans* EPN-1 contains an ENTH domain, two Ubiquitin interacting motifs (UIMs), several AP2-binding motifs, in addition to two clathrin-binding consensus sequences and four NPF motifs (Dr. MG Malabarba, personal communication). The conservation of the domains identified in the Epsin protein family suggests that the *C. elegans* homologue would also display an endocytic function.

F15C11.2, an as yet uncharacterized *C. elegans* gene is thought to be homologous to mammalian PLIC proteins (Ubiquilins), in particular to PLIC-2. Mammalian Ubiquilin has been shown to interact with the UIM domain of Eps15 and of Epsin [149]. This study confirmed the F15C11.2 (ubiquilin)-EHS-1 (eps15) interaction in *C. elegans*. A very recent study on PLIC-2, has distinguished its function from that of PLIC-1 in the negative regulation of GPCR (G-protein coupled receptor) signalling. PLIC-2 was demonstrated to inhibit the agonist-induced internalization of GPCR by delaying the receptor co-clustering with arrestins, which are adaptor proteins required for GPCR endocytosis [151]. Given the implication of mammalian

Ubiquilin in GPCR endocytosis, and the role of EHS-1 in the endocytic processes, F15C11.2 might also play a similar role in *C. elegans*.

ALX-1, the known interactor of RME-1 [101] as mentioned Section 2.4.1, was found to interact also with EHS-1 in the Y2H screenings. In addition, ALX-1 was shown to interact with the EH domains of ITSN-1 and REPS-1 by the criss-cross transformations. The mammalian homologue of ALX-1, Alix, was also identified as an interactor of CIN85/SETA/Ruk [152], which regulates the endocytosis of EGFR through its interaction with Endophilin. Endophilin interacts with dynamin, and is essential for the negative membrane curvature in vesicle formation from the plasma membrane. Endophilin itself also interacts with Alix [153]. Therefore, the interaction between ALX-1 and EHS-1 identified in this study, may point to a role for them in vesicle formation in C. elegans. EHS-1 has also previously been shown to play a role in synaptic vesicle recycling together with dynamin, dyn-1 [19].

C. elegans Disabled protein, DAB-1 was identified to interact with ITSN-1, EHS-1 REPS-1, and RME-1 in this study. While DAB-1 in *C. elegans* is a unique component of the Dab family, two members exist in mammals. Disabled-1 (Dab1), plays a role in Reelin signalling, and affects the positioning of cells during the neuronal development [154]. On the other hand, Disabled-2 (Dab2) has been described as a cargo-selective adaptor protein for endocytosis of the lipoprotein receptor family [155, 156].

16.2 EH interactors in Actin cytoskeleton dynamics

CAS-1, which is a novel interactor of EH proteins identified in this study, has not yet been characterized in *C. elegans*. However, its sequence is homologous to Cyclase-

associated protein1 (Cap1) in yeast, *Drosophila* and human. Cap1 has been implicated in vesicle trafficking and endocytosis as well as actin cytoskeletal organization [157, 158]. The total sequence homology of 38%, but observed throughout the sequence of the entire protein between CAS-1 (especially the shorter isoform b and c) with the yeast and the mammalian Cap1 suggests that CAS-1 could share the same functions in *C. elegans*, although this still needs to be explored. The interaction of Cap1 with the EH proteins has not been reported and analysis of this interaction in mammalian system could extend the link between the EH protein and the cytoskeleton dynamics.

F15C11.2, an EH interactor that may be involved in GPCR endocytosis as described in Section 16.1, may also play a role in actin dynamics, considering the functions demonstrated by its mammalian homologues. PLICs have been identified as a partner of integral membrane protein CD47, which is involved in adhesion and migration and also as a component of the actin cytoskeleton [159]. Consistent with this finding, PLIC-1 was found to play a role in the regulation of Gi-coupled receptor activity, which has been implicated in the migration of cells in chemotaxis [160], in addition to another study, which implicated PLIC-2 in the endocytosis of GPCR described in Section 16.1. Although the interaction between Eps15 and PLIC-2 has been reported [151], no data are available regarding a possible role in actin organization or in the connection of PLIC-1/2 with other EH proteins.

16.3 EH interactor involved in Receptor maturation

LIN-10 was shown to interact with the EH domains of EHS-1, ITSN-1 and REPS-1 in this study. LIN-10 was demonstrated as a part of LIN-2/LIN-7/LIN-10 complex, which plays a role in the basolateral localization of EGFR/LET-23 [140, 161].

Intriguingly, LIN-10 in neurons was recently shown to direct the localization of glutamate receptor, GLR-1 in *C. elegans*. [162, 163] These findings suggest that LIN-10 is involved in the receptor sorting process in a cell-type specific manner. It is the first time that LIN-10/Mint-1 has been proven to interact with the EH proteins and it will be of interest to further investigate the role of the EH network in receptor localization in mammalian cells.

16.4 EH interactor involved in ligand sorting

EPN-1, identified to interact with ITSN-1, EHS-1, and REPS-1 in this study, is a *C. elegans* homologue of Epsin protein family, as mentioned in the earlier section 16.1. EPN-1 in *C. elegans* was reported to participate in the maturation of the ligand for Notch receptor, GLP-1 in the development of the germline distal tip cells [164]. Apart from this study, most of what we know about the Epsin family comes from studies on the *Drosophila* homologue, Liquid facets (lqf). Lqf plays a role in the endocytic processes that take place in the signal-sending cells in Notch pathway [164, 165], which is consistent with the role of EPN-1 in promoting the Notch signalling, as mentioned earlier in this subsection. The ligand maturation process in the signal-sending cells is a pre-requisite for the activation of the Notch signalling pathway. The Notch ligand Delta of DSL (Delta Serrate LAG-2) ligand family is ubiquitinated by Neuralized (in *Drosophila* and Mindbomb in Zebrafish) and undergoes subsequent dynamin-mediated endocytosis, before the ligand is re-expressed on the cell surface as activated ligand. The trans-endocytosis of Delta-bound extracellular domain of Notch receptor was shown to be necessary for the proteolytic release of the Notch intracellular domain [166]. Therefore, endocytosis is a critical process for Notch activation, because without it, the Notch ligand cannot be activated. The interactions of EH proteins with EPN-1, lend support to the fact that

endocytosis-dependent Notch ligand activation pathways in *Drosophila* and in *C. elegans* are conserved, and further confirm the involvement of the EH network in endocytosis.

16.5 EH interactors in Endocytosis of membrane receptors and ligand secretion

DAB-1, which was previously mentioned in Section 16.1, was recently shown to be a clathrin-binding protein, that is involved in the secretion of EGL-17, the fibroblast growth factor (FGF) in *C. elegans* [167]. The clathrin-binding adaptor protein function of DAB-1, its sequence alignment with mammalian homologues, and the recent reports implicating it in endocytosis [167-169] indicate that *C. elegans* DAB-1 is more similar to the adaptor-protein Dab2, than to Dab1. To date, evidence on possible interactions between DAB family and EH proteins in mammals are not available.

Another putative component of the secretory pathway that was found to interact with EHS-1 and ITSN-1 in this study is TFG-1 (Trk-fused gene-1). TFG-1 is referred to as COPII complex, which takes part in the protein traffic from ER to Golgi apparatus [170].

Interestingly, in support of the functions of DAB-1 and TFG-1 in protein trafficking, both proteins were recently identified in a genome-wide RNA*i* screening for genes involved in membrane traffic in *C. elegans* [170].

16.6 EH interactors in Early endosome trafficking

SEL-5 is an NPF motif-containing protein, which had been predicted to be an EH interactor, as mentioned in the section 14.2 of this discussion, and was confirmed to interact with REPS-1 in this study. SEL-5 was initially identified in a screening for the suppressor/enhancer of LIN-12, one of the Notch receptors in *C. elegans* [144].

The yeast homologues of SEL-5, Ark1p and Prk1p (Pak1p) are Serine/Threonine (S/T) kinases. Prk1p was initially shown to regulate the actin cytoskeleton organization by modulating yeast EH domain-containing proteins Pan1p (a hypothetical homologue of intersectin) and End3p (an yeast homologue of Eps15) [171]. More recently, the function of Prk1p was further elaborated as it was shown that the phosphorylation of Pan1p by Prk1p inhibits the fusion of vesicles to endosomes by inhibiting the Arp2/3-mediated actin polymerization on endocytic vesicles [172]. Of note, the findings that Prk1p, the yeast homologue of SEL-5 phosphorylates an EH-domain containing protein Pan1p lends confidence to the validity of the interaction between SEL-5 and the EH domain of REPS-1 identified in this study.

Further implication of SEL-5 in early steps of endocytic trafficking comes from the knowledge on its mammalian homologue. The S/T kinase domain of Cyclin C-associated Kinase 1 (GAK1) is thought to be the most similar to the kinase domain identified in the N-terminal portion of SEL-5 [144]. GAK1 was is a co-factor of the chaperone protein Hsc70 during uncoating of clathrin-coated vesicles during endocytosis [173]. However, the sequence homology between SEL-5 and GAK is restricted to the S/T Kinase domain in their amino-terminal region, and while the region similar to auxillin that is attributed to the clathrin-uncoating activity is not conserved. Therefore, caution is warranted in suggesting that SEL-5 might act in a similar way to GAK1 as a clathrin-uncoating protein.

16.7 EH interactors in MVE/MVB sorting

ALX-1, which was identified as an interactor of EHS-1, RME-1 and ITSN-1 in this study and was the only protein that had already been demonstrated to interact with

RME-1, as discussed in Section 14.1. ALX-1 takes part in the post-internalization trafficking of the Notch receptor LIN-12 [174]. The knock-down of ALX-1 in *C. elegans* resulted in the accumulation of internalized LIN-12 in intracellular puncta, demonstrating that ALX-1 is not required for internalization of LIN-12, but rather that it plays a role in the trafficking of LIN-12 at a step subsequent to the internalization. Furthermore, the yeast homologue of ALX-1, Bro1p and the mammalian Alix were both shown to promote MVB sorting pathway as they were shown to interact with the endosomal complexes ESCRT-I and ESCRT-III [175, 176] To date, the interaction of mammalian alix with the EH proteins has not been reported.

Intriguingly, mammalian Alix has also been described as an adaptor protein that controls the function of lysobiphosphatidic acid (LBPA) in the biogenesis of endosomes [177]. LBPA is a phospholipid found in the late endosomes. Additional evidence suggests that Alix is able to regulate the intracellular positioning of endosomes in an actin cytoskeleton-dependent manner [178].

16.8 EH interactors in Receptor recycling

As mentioned in the previous section 16.7, ALX-1 was recently reported to be an interactor of RME-1 (this was confirmed in this project), and to function in receptor recycling from the endosomes to the plasma membrane [101]. EHS-1, another EH protein that was demonstrated in this project to interact with ALX-1 was shown to play a role in synaptic vesicle recycling, suggesting a role for ALX-1 in this process.

16.10 The EH proteins and the EH interactors in the nucleus

Although Eps15 and Eps15R are classically found to interact with AP-2 and clathrin and are localized at the coated pits, a significant fraction of Eps15R in the nucleus has

been reported [179, 180]. Eps15R lacks the Nuclear Export Signal (NES), which is found in the C -terminal of Eps15. The sequence homology between the human Eps15 and its C. elegans homologue EHS-1 is not found in their C-terminal regions, and the sequence particularly rich in Leucine residues is not found in EHS-1, as in the case of Eps15R. However, the function of Eps15R or of EHS-1 in the nucleus, is yet to be elucidated.

Mammalian Epsin (which is an established interactor Eps15), and its *C. elegans* homologue EPN-1 (that was shown to interact with several *C. elegans* EH proteins in this study), have been found localized in the nucleus together with Eps15 and CALM (clathrin assembly lymphoid myeloid leukaemia). The ENTH domain of Epsin has been shown to interact with a PLZF (promyelocytic leukaemia Zn2+ finger protein) transcription factor [181], a nuclear protein and therefore further supporting the nuclear localization and a possible nuclear function of Epsin.

One of the three specific interactors of EHS-1 identified in this study is the uncharacterized protein C50C3.8. This protein is similar to the *Drosophila* protein roadkill (rdx), which is expressed in response to Hedgehog (Hh) signaling. Rdx is localized in the nucleus and thought to act as an adaptor protein for Cullin 3, an E3 ubiquitin ligase that promotes the degradation of the transcription factor, Cubitus interruptus (Ci) [182]. Roadkill homologue 1/HIB (Hedgehog induced BTB domain containing protein) is found also conserved in human, and the negative feedback loop that serves to attenuate Hh signalling is thought to be conserved.

It is interesting to note that the sequence of C50C3.8 contains a region recognized as a POZ/BTB domain. This domain is found common in transcription factors such as

PLZF and Kaiso. PLZF, as mentioned earlier in this section, is an interactor of ENTH domain in Epsin, whereas Kaiso interacts with the ENTH-like armadillo repeat domain of catenin p120 [183]. Even though Rdx is an adaptor protein involved in the degradation of a transcription factor and does not seem to act as a transcription factor (such as, instead, PLZF or Kaiso), the presence of a POZ/BTB domain in C50C3.8 further suggests that this uncharacterized protein may be a nuclear protein.

16.11 EH interactors in Protein degradation

F15C11.2 was shown to interact with ITSN-1, EHS-1 and REPS-1 in this study. Its possible functions in endocytosis and in the regulation of the actin cytoskeleton dynamics implied by the mammalian homologues Ubiquilin/PLICs have been discussed previously in Section 16.1 and 16.2. An additional possibility for F15C11.2, which is still uncharacterized in *C. elegans*, is that it may be involved in proteasome degradation as inferred from the function of its yeast homologue Dsk2p. Dsk2p, the single yeast PLIC protein, was shown to interact with poly-ubiquitin chains and with the proteasome[184]. Furthermore, Dsk2p was also identified in the genetic screen for components essential in the ER-associated degradation of mal-folded proteins.

BE0003N10.3 is another uncharacterized protein identified in this study as an interactor of ITSN-1 that may be predicted to play a role in protein degradation. BE0003N10.3 was identified as the only specific interactor of ITSN-1. The amino acid sequence contains similarity to proteins in *Drosophila* and in mammals that are involved in ubiquitin ligase activity, such as the F-box only protein 11 (FBX11) family. These proteins are a part of E3 ubiquitin ligase complex, SCF (Skp1-Cul1-F-

box) complex. FBX11 contains different domains such as cyclin-like F-box domain and CASH (carbohydrate-binding and sugar hydrolysis), Pectin lyase-fold and Zinc finger domain. The sequence similarity between FBX11 and BE0003N10.3 is found in the CASH domain and pectin lyase-fold region, rather than in the F-box domain. Therefore, whether BE0003N10.3 is a true homologue of FBX11, as stated in the Wormbase, is still to be investigated.

16.12 The EH interactors at the cross-roads of signalling pathways

16.12.1 At the cross-roads of EGFR-Ras signaling and Notch signaling pathways

The study on the role of ALX-1 in Notch/LIN-12 down-regulation, which was previously mentioned in Section 16.7, also shed light on the cross-talk between Notch pathway and EGFR-Ras-MAPK pathway that occurs during vulval development in *C. elegans* [174]. Cell-fate determination of vulval precursor cells (VPC) depends on the activation of either the EFGR-Ras-MAPK signaling cascade or the LIN-12 signaling pathway in each VPC. It was shown that EGFR-Ras-MAPK activation in the primary VPC promotes the endocytosis and the subsequent degradation of LIN-12. The degradation of LIN-12 in the primary VPC in turn activates LIN-12 signaling pathways in the neighbouring secondary VPC cells. ALX-1 was found to play a role in LIN-12 degradation after its internalization in the primary VPC cells.

Another EH interactor identified in this study that may be involved in the LIN-12 pathway of vulval development is LIN-10. LIN-10 has been commented on earlier in Section 16.3 for its function in receptor maturation of EGFR/LET-23 and GluR/GLR-1. The *C. elegans lin-10* mutant strain manifests a vulvaless phenotype

[185], commonly seen for genes involved in Notch/LIN-12-mediated cell-fate determination.

16.12.2 At the cross-roads of EGFR/Ras signalling pathway and actin dynamics

LIN-10 was identified in this study as an interactor of three EH proteins, ITSN-1, EHS-1, and REPS-1. LIN-10 forms a trimeric complex with LIN-2 and LIN-7 that determines the basolateral localization of EGFR/LET-23, as described previously in Section 16.3 [139]. Recently, EPS-8, an actin-binding protein, was reported to be part of LIN-2/LIN-7/LIN-10 complex [186]. Mammalian Eps8 was initially identified as a substrate of the tyrosine kinase domain of EGFR, and was suggested to play a role in mitogenic signalling [187]. Subsequently, Eps8 was shown to interact with F-actin and both the mammalian and the nematode Eps8 were demonstrated to have actin barbed end-capping activity [188, 189]. The finding that EPS-8 interacts with the heterotrimer LIN-2/LIN-7/LIN-10 implicates LIN-10 in coupling the actin organization and the activation of EGFR signalling via this multimeric complex.

CAS-1 is another EH interactor identified in this study that may act in between EGFR/Ras signalling pathways and actin re-arrangement. Although no specific function of CAS-1 has been reported in *C. elegans* to date, its homologues that are implicated in actin organization have been discussed in Section 16.2. Cap1p/Srv2, the yeast homologue of CAS-1, binds to Adenylyl Cylase (AC) and acts as an effector of Ras signalling pathways (reviewed in [190]). AC is an enzyme responsible for the formation of cyclic-AMP (cAMP), a key effector in Ras signalling pathways. Cyclase-associated proteins (CAPs) are thought to act as a link between Ras signalling pathway and actin polymerization.

16.12.3. At the cross-roads of Apoptosis, Ras activation and actin dynamics

The CAP proteins, the protein family of CAS-1 as described in the previous section 16.13.2, were recently reported not only as a molecular link between the actin dynamics and Ras-activation, but also as a link between these processes and apoptosis [191]. Ras signalling activation was found to correlate with the accumulation of reactive oxygen species (ROS), the aggregation of F-actin, and the activation of apoptotic pathway.

Fascinatingly, Gourlay and Ayscough created the accumulation of F-actin in yeast stationary cells by using the yeast mutants of End3p, the Eps15 homologue, and an SH3 domain-containing protein Sla1p. End3p and Sla1p interact with each other in a heterotrimeric complex with another EH protein Pan1p. The trimeric complex consists of multiple EH domains and SH3 domains, a structure that is similar to that of the heterodimer formed between the mammalian Intersectin and Eps15. It was demonstrated that in *end3* and *sla1* mutants, CAP/Srv2p is required for the co-localization of the GTP-bound Ras protein on F-actin aggregates, as well as for the ROS accumulation, thus for the subsequent apoptosis. The study placed CAP proteins at the intersection of Ras proteins on actin dynamics and the activation of apoptosis. Furthermore, the observation that the yeast homologue of CAS-1 is needed for the apoptosis of cells which lack an EH protein (End3p) and its interactor (Sla1p), supports the finding in this study that CAS-1 is in the EH network.

16.12.4 EH interactors in EGFR signalling and apoptosis

Alix, the mammalian homologue of ALX-1, which was shown to interact with RME-1 by others and with other EH proteins in this study, is also thought to be involved in

apoptosis. The other functions of Alix proteins such as those in MVB sorting, endosomes biogenesis and recycling have been discussed previously in Section 16.7 to 16.9. Alix was originally identified as an interactor of apoptosis-linked gene 2 (ALG-2), a gene that was shown to be necessary for cell death [192]. Subsequently, Alix was directly implicated in apoptosis as it was shown to induce cell death when overexpressed. Interestingly, the C-terminal region of Alix is required for the binding with ESCRT complex, and the interaction between Alix and ESCRT complex is necessary for the inhibition of cell death [193, 194].

It would be interesting to investigate whether the interactions between EH proteins and Alix could affect the further interactions between Alix with other proteins, such as ALG-2. Interactions mediated by Alix could determine the EGFR signalling to continue or could instead activate the apoptotic pathway. Such studies would allow us to better understand the role of EH proteins as adaptor proteins that transport their interactors, such as Alix, to specific locations within the cell to determine particular outcomes (for example, growth vs. cell death).

17. The EH network implicated in diseases

The EH domain was initially recognized as the repeated domains in the N-terminal domain of Eps15, a protein that was identified as the substrate of the tyrosine kinase domain of EGFR. Increased activation levels of EGFR signalling in quiescent cells promotes cell cycle re-entry; such increased and uncontrolled cell division is the first step of tumorigenesis.

The process of endocytosis is an important mechanism by which the continued activation of EGFR is attenuated by receptor internalization. The EH domains

located in Eps15 and in other endocytic proteins mediate protein-protein interactions required not only for endocytosis at the plasma membrane, but at different steps of intracellular trafficking. The new interactors of EH domains identified in this study support the view that the EH network proteins function at diverse level of protein trafficking downstream of EGFR signalling, ensuring the efficient attenuation of EGF signal. The various functions of EH network proteins in signalling pathways other than EGFR, that have also been implicated in oncogenesis, such as Notch signalling have been examined in the previous section 16.

17.1 The EH proteins and their interactors in HIV-1 infected cells

The usual mechanism of the release of virus is by cell lysis of the host cells. In contrast, retroviruses, such as HIV-1, release their virions by budding from the membrane of the host cells. Some EH interactors, such as Hrb and Hrbl and Alix participate in the protein trafficking required for the replication of HIV-1. The mammalian Eps15 family proteins interact with Hrb and Hrbl (Hrb-like), which are co-factors of viral Rev proteins [195]. Hrb proteins interact with Rev proteins, encoded by the HIV-1, and assist the nucleo-cytosolic shuttling of the viral RNA in a necessary process for the assembly of viral particles.

Another EH interactor reported to assist the virus budding process is Alix, the mammalian homologue of ALX-1 that was identified in this study and by Shi *et al* [101]. Alix, together with TSG101, are the host endosomal proteins that are "hi-jacked" during the HIV-1 infection. Gag polyprotein encoded by HIV-1 orchestrates the viral assembly and the budding. There are several motifs referred to as the "late domains" found on the retroviral protein; P(T/S)AP, PPxY, and YPx_nL (where x is any residue and x_n is any sequence). "Late domains" are found in the viral proteins

that are expressed after the early proteins, at a stage of infection promoting the release of the virions [196]. The YPx_nL-type domain was shown to bind the V domain of Alix, which was found to form a conformation similar to a letter V [196]. Alix interacts with both TSG101 in ESCRT-I complex and CHMP4 in ESCRT-III complex. The structural study on Alix showed that the Bro1 domain of Alix that is responsible for the interaction between Alix and an ESCRT III complex protein, CHMP4, is crucial for the function of Alix in virus budding. A recent study on the structure of Alix presented a banana-shaped Alix [196], which supports its role in inducing the membrane curvature during virus budding.

Recently it was shown that the HIV-1 gp41, which is an important player in the fusion between the viral and the target cell membranes, binds to the Epsin NPF motifs and that a synthetic peptide containing the Epsin-1 (470-499) sequence blocks the entry of HIV-1 virions into SupT1 T cells via the endocytosis pathway. These results suggest that interaction between Epsin and the gp41 core, which may be present in the target cell membrane, is probably essential for endocytosis of HIV-1, an alternative pathway of the HIV-1 entry into the target cell [197]. The participation of EH proteins in the process required for the entry of HIV-1 into cells is so far completely unexplored.

17.2 EH proteins implicated in neurodegenerative diseases

As mentioned in the introduction of the EHD proteins (Section 2.4.6), an EHD family protein, Pincher is involved in the retrograde signalling of NGF in the neurons isolated from postnatal day 1 and embryonic (E18) rat [124]. The retrograde signalling may play a critical role in the pathogenesis of neurodegenerative diseases. The hallmarks of neurodegenerative diseases are the loss of neurons and synapses. The failure to

transport the signal from the axonal terminals to the cell bodies results in the loss of the cell survival signal, therefore resulting in the degeneration of neurons.

Proteins that are implicated in neurodegenerative diseases, such as Amyloid Precursor Protein (APP), which gives rise to beta-amyloid peptides (Aβ), and poly-glutamine (polyQ)-containing proteins, have been shown to be neurotoxic. Recently, the EH protein Intersectin was reported to increase the formation of aggregates by mutant huntingtin protein (Htt) through activation of the c-Jun-NH(2)-terminal kinase (JNK)-MAPK pathway [210]. Conversely, silencing ITSN or inhibiting JNK attenuated aggregate formation. Using a *Drosophila* model for polyQ repeat disease, Intersectin was found to enhance polyQ-mediated neurotoxicity, suggesting a broader involvement of Intersectin in neurodegenerative diseases through destabilization of polyQ-containing proteins. Moreover, human Intersectin gene is located on chromosome 21, and its overexpression in Down Syndrome and its potential role in the early onset of Alzheimer's Disease (AD) pathogenesis observed in DS patients has been discussed in a recent review [198].

In addition, AD is frequently mentioned in conjunction with the mammalian homologues of some of the proteins that were identified in this project as the EH interactors. In the following section the implication of EH interactors in AD will be discussed.

17.2.1 The EH interactors implicated in Alzheimer's Disease

Alzheimer's Disease is one of the most common neurodegenerative diseases that manifests in dementia in the elderly. The mechanisms that are involved in the pathology of AD are very complex, though it is evident to date that the degeneration of neurons is

preceded by the failure of efficient synaptic transmission [199]. Extracellular accumulation of fibrillar Aβ peptide forming the amyloid plaques is one of the hallmarks of AD and the neurotoxicity of Aβ accumulation has been demonstrated. Not only extracellular but more recently, intracellular Aβ is also thought to contribute to the increased neuronal death. Aβ is a peptide of 39 to 43 residues generated from the proteolysis of APP by secretases and other proteases.

In this context it is important to note that some EH interactors (EPN-1, ALX-1 and SEL-5) are involved in Notch signaling pathway, which recently received attention for its link with AD. Notch receptor has been shown to interact with APP and it was shown that Notch1 and APP are able to trans-activate each other's target genes [200]. Furthermore other EH interactors (Lin-10/Mint-1, Disabled and PLICs/Ubiquilin), have been implicated in AD, as reported below.

LIN-10 and DAB-1 are both phosphotyrosine binding (PTB)-domain containing proteins. The PTB domains of the mammalian LIN-10 and DAB-1 interact with APP or with the members of APP family [201, 202]. The Lin-10 mouse orthologue promotes the stability of APP, and thus downregulates the production of A-beta (Aβ) production [203, 204]. In comparison, mouse Dab1 (mDab1) has been implicated in increasing the production of Aβ [203]. The PDZ domain of Lin-10 interacts with Presenilin-1, which is a component of γ-secretase, the protease responsible for the cleavage of APP. The interaction between X11/Lin-10 and Presenilin-1 is suggested as the mechanism by which X11 promotes the stability of APP.

PLICs/Ubiquilin was also identified as an interactor of presenilin [205], and it has been implicated in AD by several studies. For example, Ubiquilin was shown to be localized

in the neurofibrillary tangles and Lewy bodies, which are found in AD affected brains [205]. The knockdown of Ubiquilin accelerated the maturation and trafficking of APP from the intracellular compartments to the cell surface [206]. More recently, the evidence of genetic interaction between ubiquilin and presenilin in *Drosophila* has also been reported [207, 208].

Intriguingly, the UBL domain of PLIC-1 interacts with the UIM domain of Eps15, and both PLIC-1 and Eps15 are recruited to the poly-glutamine (polyQ) aggresomes [209]. PolyQ aggresomes are different from Aβ plaques. PolyQ are involved in another type of neurodegenerative diseases often referred to as prion diseases, in which plaque formation is characteristic. In addition, the toxic polyQ peptide is approximately 42 residues, similar to the length of Aβ in the range of 39-43 (as specified earlier in this section), suggesting that a similar mechanism is involved in causing the neurotoxicity.

18. Closing Remarks

In summary, the EH proteins and the protein-protein interactions mediated by the EH domains are involved in the regulation of numerous intricate intracellular cross-talking signalling pathways.

The EH network seems to provide a web of platforms through which different signalling molecules travel. Once the signalling molecules are brought together with the adaptor proteins, signalling events such as phosphorylation or ubiquitination can occur. The fact that the EH interactions act as transient platforms for divergent pathways is supported by the finding that some adaptor protein such as ALX-1, EPN-1, LIN-10, DAB-1 or PLIC-1, identified in this screening as EH interactors often act at intersections of different signalling pathways.

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